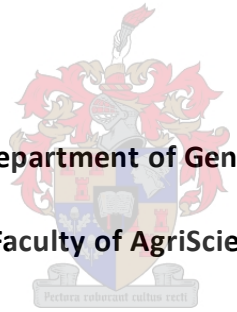
Improvement of rust resistance and FHB resistance QTL in wheat through the application of an integrated biotechnology approach

by

Schalk Willem Meintjes



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DECLARATION

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March 2017

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PREFACE

Science Contributions during Master Candidature (2015-2016)

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2. Local conference poster

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3. International conference presentation

Aletta Ellis[1], Rayganah Rhoda[1], Lezaan Hess[1], Kim Coetzee[1], S.W. Meintjes[1], Jabulani Mthembu[1], Cebekhulu Nondumiso[1], Wandile Ngcamphalala[1] and Willem Botes[1]. A collaborative approach to pre-breeding in South Africa. *13th International Wheat Genetics Symposium*. April 2017. Tulln, Austria.

ABSTRACT

The international demand for wheat production is placing pressure on breeders to increase and develop elite cultivars which are adapted to meet specific criteria such as high grain quality and high yield potential (Francki and Appels, 2002). Unfortunately these cultivation and agronomical demands are not the only factor for breeders to think about when it comes to food security. More specifically, abiotic and biotic stressor influence food security significantly. Each year 10-16 % of global harvest is lost due to plant diseases (Oerke, 2006). Based on a Molecular Plant Pathology survey taken in 2012, the top 10 fungal pathogens affecting crop production was taken and *Puccinia* species where listed third, followed by *Fusarium graminearum* (FHB) in fourth place (Dean *et al*., 2012). The wheat rust fungi (leaf, stem and stripe rust) have become economical important diseases worldwide. *Puccinia* have the ability to mutate, migrate and recombinate, these abilities are predominantly the reason why wheat rust epidemics are a serious concern in wheat growing areas around the world (Sing *et al*., 2002). FHB occurs in a number of wheat growing regions (Asia, Australia, Canada, Kenya, Europe, North and South America and South Africa) (Waalwjik *et al*., 2003; Guo *et al*., 2008; Karugia *et al*. 2009). The first FHB report on wheat in South Africa was in 1980 in the North-West Province (Scott *et al*., 1988). FHB produces mycotoxins that contaminate grain, causing lower yield and quality (Leonard and Bushnell, 2003). The challenge however is to pyramid FHB QTL and rust genes into one cultivar for durable resistance.

The aim of the study was to pyramid rust genes and FHB QTL in to one genotype by performing the male sterility mediated marker assisted recurrent selection scheme (MS-MARS). A segregating F_1 population was provided for the MS-MARS cycle 1 from an existing pre-breeding nursery. Molecular markers were successfully implemented to determine the gene frequenciesof rust genes (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34,Lr37* and *Lr19*) in the segregating F_1 population.The cross was between a rust male donor and a FHB male donor population to produce two independent sub-populations in MS-MARS cycle 1. The gene frequencies of the rust male donors were known. The gene frequencies of the FHB male donor population were

successfully determined through molecular characterization. The FHB QTL of interest was (*Qfhs.ifa-5A*, *7AQTL* and *Qfhs.ndsu-3BS*).

The gene frequencies of 40 %, 92 %, 0.8 %, 48 %, 1.68 %, 69 % and 9.45 % were observed for *Sr2*, *Sr24*, *Sr26*, *Sr31*, *Lr19*, *Lr34* and *Lr37* respectively in the segregating F_1 base population of cycle 1. The gene frequencies of 83.30 %, 17 % and 66.67 % were observed for *Qfhs.ndsu-3BS*, *7A QTL* and *Qfhs.ndsu-5A-1* respectively in the FHB male donor population.

Future studies will include the development of a double haploid population with pyramided rust genes and FHB resistant QTL. Determine the effect FHB resistance have on the bread baking properties. Phenotyping the presence of rust and FHB QTL through inoculating the population with rust and fusarium isolates.

OPSOMMING

Die internationale aanvraag na koringproduksie plaas druk op koringtelers vir 'n toename en die ontwikkeling van elite kultivars wat aangepas is om hoër graankwaliteit en obrengste te lewer (Francki en Appels, 2002). Ongelukkig is die vraag na hoër en beter koringobrengste nie die enigste probleem vir tellers as dit kom by voedselsekuriteit nie. Meer spesifiek, abiotiese en biotiese faktore speel ook 'n beduidende rol in voedselsekuriteit. Elke jaar word 10-16 % van die wêreld se koring produksie gekelder deur plantsiektes (Oerke, 2006). 'n Molekulêre Plant Patologiese opname in 2012, het aangedui dat ten opsigte van die top 10 patogene wat gewasproduksie benadeel, *Puccinia* species was algeheel in die derde plek, gevolg deur *Fusarium graminearum* (FHB) in die vierde plek (Dean *et al*., 2012). Die koringroes swamme (blaar-, stam- en streep-roes) het 'n ekonomiese belangrike siekte geword wêreldwyd. *Puccinia* besit die vermoë om te muteer, migreer en te kan rekombineer. Hierdie vaardighede is hoofsaaklik die rede hoekom koringroes epidemies so 'n ernstige probleem is in die koringproduksie areas regoor die wêreld (Singh *et al*., 2002). FHB kom voor in verskeie koring produksie streke (Asië, Australië, Kanada, Kenya, Europa, Noord en Suid Amerika en Suid-Afrika) (Waalwjik *et al*., 2003; Guo *et al*., 2008; Karugia *et al*. 2009). Fusarium is vir die eerste keer op koring in Suid-Afrika waargeneem gedurende 1980 in die Noord-Wes Provinsie (Scott *et al*., 1988). FHB vervaardig mikotoksiene wat koring kontamineer en so doende die kwaliteit en obrengs verlaag (Leonard and Bushnell, 2003). Die uitdaging bestaan dus om FHB QTL en roes gene te stapel in een kultivar vir duursame weerstand.

Die doel van die studie was om roes gene en FHB QTL te stapel deur die manlike steriele bemiddelde merker assisterende herhalende seleksie skema uit te voer (MS-MARS). 'n Segregerende F_1 populasie was verskaf vir die MS-MARS siklus 1 vanaf 'n bestaande voor-telende kwekery. Molekulêre merkers was suksesvol geimplimenteer vir die bevestiging van geen frekwensies vir die volgende roes gene (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34,Lr37* and *Lr19*) in die segregerende F_1 populasie. Die kruisings was tussen 'n manlike roes donor en manlike FHB donor populasies om twee onafhanklike sub-populasies te produseer in MS-MARS siklus 1. Die manlike

roes donor geen frekwensies is bepaal. Slegs die manlike FHB donor geen frekwensies was bepaal deur molekulêrekarakterisering. Die FHB QTL van belang was (*Qfhs.ifa-5A*, *7AQTL* and *Qfhs.ndsu-3BS*).

Die geen frekwensies van 40 %, 92 %, 0.8 %, 48 %, 1.68 %, 69 % and 9.45 % was waargeneem vir *Sr2*, *Sr24*, *Sr26*, *Sr31*, *Lr19*, *Lr34* and *Lr37* respektiewelik vir die segregerende F_1 basis populasie van MS-MARS cycle 1. Die geenfrekwensies van 83.30 %, 17 % and 66.67 % was bepaal vir *Qfhs.ndsu-3BS*, *7A QTL* and *Qfhs.ndsu-5A-1* respektiewelik vir die manlike FHB donor populasies.

Toekomstige studies sal fokus op die ontwikkeling van verdubbelde haploïdes met gestapelde roes gene en FHB weerstand QTL's. Evalueer die die effek wat FHB weerstand het op die bak kwaliteit van brood. Bevestig die teenwoordigheid van roes gene en FHB QTL's deur die populasie te inokuleer met roes- en fusarium isolate.

LIST OF ABBREVIATIONS

%	Percentage
@	At
∞	Infinity
3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
4-ANIV	4-acetyl-nivalenol
AFLP	Amplified Fragment Length Polymorphism
$AgNO_3$	Silver Nitrate
APR	Adult Plant Resistance
APS	Ammonium persulfate
bp	Base pairs
≥	Bigger or equal
CAF	Central analytical facility
CE	Capillary electrophoresis
˚C	Degrees Celsius
CHA	Chemical hybridizing agents
CIMMYT	*Centro Internacional de Mejoramiento de Maiz y Trigo*
cm	Centimeter
CO_2	Carbon dioxide
Conc	Concentration
CMS	Cytoplasmic male sterility
CTAB	N-Cetyl-N, N, N-trimethyl Ammonium Bromide
DArT	Diversity Arrays Technology

DH	Doubled Haploid
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
dpi	Days prior inoculation
$	Dollar
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethane sulfonate
EtBr	Ethidium Bromide
et al	Number of people
F	Forward primer
F_1	First generation
F_2	Second generation
F_3	Third generation
f. sp.	Forma specialis
FHB	Fusarium head blight
g	Gram
gDNA	Genomic Deoxyribonucleic Acid
GMS	Genetic male sterility
h	hour
HR	Hypersensitive response
H_Z	Hertz
Inc.	Incorporation

Kg	Kilograms
L	Liter
LED	Light Emiting Diode
Lr	Leaf rust resisance gene
ltn	Leaf tip necrosis
Ltd	Limited Liability
M	Molar
MAS	Marker assisted selection
Mb	Mega bases
min	Minutes
ml	Millilitre
mm	Millimeter
mm^2	Square millimeter
mM	Millimolar
mm^3	Cubic millimeter
MS-MARS	Male Sterility Mediated Marker Assisted Recurrent Selection
Mt	Million tons
n	Haploid
2n	Diploid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
$ng.\mu l^{-1}$	Nanogram per microlitre
NIV	Nivalenol

PAGE	Polyacrylamide gel electrophoresis
Ph1	Pairing homoeologous 1
PBC	Pseudo black chaff
PCR	Polymerase chain reaction
pH	Percentage hydrogen
Pty	Ltd Propriety Limited
QTL	Quantitative Trait Loci
R	Reverse primer
RAPD	Random Amplified Polymorphic DNA
RFLPs	Restriction fragment length polymorphisms
R-gene	Race specific resistance gene
RH	Relative Humidity
Rpm	Revolutions per minute
RSA	Republic of South Africa
rxn	Reaction
SCAR	Sequence Characterized Amplified Region
sec	Seconds
SNP's	Single nucleotide polymorphisms (SNP)
spp.	Species pluralis
Sr	Stem rust resistance gene
SSD	Single seed descent
SSR	Simple Sequence Repeat
STR	Simple tandem repeats
SU-PBL	Stellenbosch University Plant Breeding Laboratory

Ta	Temperature
TBE	Tris/Borate/EDTA
TMED	N, N, N‘, N‘‘-Tetramethyl ethylene diamine
Tris-Cl	Tris-chloride
UV	Ultra Violet
µl	Microlitre
µm	Micrometer
V	Volt
V5	Version 5
v/v	Volume per volume
w/v	Weight per volume
Yr	Stripe rust resistance gene

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Chapter 1 INTRODUCTION

Wheat (*Triticum Aestivum* L.) provides the basic carbohydrates that are being consumed by more than 4.5 billion people in 94 third world countries (Braun *et al*., 2010). The global increase of the human population puts strain on wheat producing regions to increasing wheat production with 60 % by 2050 (Rosegrant Agcaoili, 2010; Singh and Trethowan, 2007 and Singh *et al*., 2007). To meet these demands, the clearing of land or land intensity, could help to achieve the production demand. Land clearing however can cause problems; it contributes a quarter of global greenhouse gas (GHG) emissions, but results in habitat and niche fragmentation and will influence biodiversity significantly (Godfray *et al*., 2010; Burney *et al*., 2010). Leaving only one alternative; land intensification that will focus on better agronomical practises, lower input cost and superior crop cultivars (Tilman *et al.,* 2011).

Unfortunately these cultivation and agronomical demands are not the only factor. More specifically, abiotic and biotic stressor influence food security significantly. Each year 10-16 % of the global harvest is lost due to plant diseases (Oerke, 2006). Based on a Molecular Plant Pathology survey taken in 2012, the top 10 fungal pathogens affecting crop production was taken. *Puccinia* species where listed third, followed by *Fusarium graminearum* (FHB) in fourth place. These two fungal pathogens are of significant concern for wheat breeders (Dean *et al*., 2012). *Puccinia* species have the ability to mutate, migrate and survive on alternative hosts (Kolmer 2005). Ug99 (TTKSK) is an excellent example of the mutation ability of a disease. This stem rust race was first established in East Africa in 1999. By 2007, Ug99 have spread to the Islamic Republic of Iran and in 2012, a new virulent Ug99 strain was found in South African borders (FAOSTAT, 2014). TTKSK is significantly virulent to a range of wheat cultivars. In 2013, 18 000 hectares of wheat crop were affected by stem rust in Ethiopia, it was caused by a new strain of stem rust TKTT- (FAOSTAT, 2014). Stem rust is not the only wheat pathogen that is important. A new Yellow rust strain with virulence to *Yr27* caused significant epidemics in Asia, Afghanistan, Azerbaijan, Ethiopia, India, Iran, Iraq, Morocco, Pakistan, Turkey and Uzbekistan (FAOSTAT, 2014).

FHB caused by *Fusarium graminearum* produces mycotoxins that contaminate grain, causing lower yield and quality (Leonard and Bushnell, 2003).The most important mycotoxins being produced is, deoxynivalenol (DON), DON derivatives, nivalenol and zearalenone which is unsafe to be consumed by people and animals. Europe and the USA have put thresholds on the maximum level of mycotoxins in their grain and food (Magan *et al.,* 2010). The negative effect of mycotoxins is that protein synthesis is inhibited when DON binds to the peptidyltransferase protein in the ribosome (Alexander *et al.,* 2011). These factors caused by FHB and wheat rusts continue to challenge breeders when it comes to the development of resistant cultivars (Waldron *et al*., 1999). The breeding strategy to obtain these resistant cultivars is now the main focus to accelerate the development process (Tester and Langridge, 2010).

The aim of the study is to pyramid rust genes and FHB QTLs into a single genotype using the MS-MARS scheme in combination with MAS and QTL validation.

The following objectives need to be achieved for the aim to be significant.

i. Molecular characterisation of the segregating F_1 base populations, FHB male donor lines and double haploids important for this study.
ii. Cross-pollinate the male sterility mediated marker assisted recurrent selection (MS-MARS) cycle 1 and 2.
iii. Perform a single seed decent (SSD) population and validate the presence of FHB QTLs by point inoculate the selected SSD lines.

CHAPTER 2: LITERATURE REVIEW

2.1. The genetic background of wheat

Sakamura (1918) and Kihara (1924) were the first to identify polyploidy levels in diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) *Triticum* species. In table 2.1 the different types of ploidy levels, species and genomes are listed. The diploid wheat is either wild like *T. urartu* and *T. boeoticum*. The diploid einkorn wheat *T. monococum* was the first domesticated wheat type from *T. boeoticum*. The domestication event happened in the Karacadag mountain range south east of Turkey (Heun *et al.,* 1997). Tetraploid and hexaploid wheat's replaced Einkorn wheat 5000 years ago (Nesbitt and Samuel, 1996 and Perrino *et al*., 1996). The tetraploid are domesticated wheat, but have a few wild wheat relatives. Durum wheat (*T. Turgidum* ssp. *durum* L.), Sanduri wheat (*T. Timopheevii* Zhuk.), Emmer wheat (*T. dicoccon*) and Polish wheat (*T. Polonicum* L.) are the domesticated wheat types and *T. araraticum* and *T. Turgidum* L. ssp. dicoccoides are the wild wheat types (Haider, 2013). The hexaploids consist of domesticated wheat types. The wheat types are; *T. Zhukovsky, T. spelta* L., *T. Compactum* Host and *T. aestivum* L. with common names zhukovsky wheat,spelt wheat, club wheat and bread wheat respectively (Haider, 2013). All the *Triticum* species are comprised of the four genomes A, B, D and G (Petersen *et al*., 2006). The A genome of bread wheat was confirmed to be transferred from*T. urartu* (Konarev *et al.,* 1974; Allaby *et al.,* 2000; Petersen *et al.,* 2006; Gulbitti-Onarici *et al.,* 2007 and Golovnina *et al.,* 2009). The B genome of tetraploid and hexaploid wheat is still unknown although hypothesized to be from *Aegilops speltoides* (Haider, 2013). The D genome is the only one with the least amount of differentiation between its progenitors (Kihara*,* 1975 and Ogbonnaya *et al.,* 2005). The D genome donor has been identified to be the Asian goatgrass *Aegilops Tauschii* Coss (Petersen *et al.,* 2006 and Pathak *et al.,* 1940). *Ae.Tauschii* is known for its wide genetic variation, which leads to the spread between various groups around the world for cultivated wheat improvement. *Ae.Tauschii* has resistance to Hessian fly (*Mayetiola destructor),* green bug (*Schizaphis graminum*) and contributes to bread-making properties of bread wheat (Hsam *et al.,* 2001). *T. aestivum* or better known as bread wheat has an

extremely large genome 17, 000 Mb (Brenchley *et al*., 2012) and 11, 660 Mb for durum wheat (Bennett and Leitch, 2010).

Table 2.1. The different types of ploidy levels, species and genomes (Feldman and Levy, 2012)

Ploidy level	Species	Relevant genomes
Diploids	*Amblyopyrummuticum*	TT
(2n = 2x =14)	*(=Ae. mutica)*	
	Aegilops speltoides	SS
	Ae. bicomis	S^bS^b
	Ag. Longissima	S^lS^l
	Ae. sharonensis	S^lS^l
	Ae. searsii	S^sS^s
	Ae. tauschii	DD
Cedar University	*(=Ae. Squarrosa)*	
Elm College	*Ae. caudate*	CC
Maple Academy	*Ae. umbellulata*	UU
Pine College	*Ae. comosa*	MM
Oak Institute	*Ae. uriaristata*	NN
	Triticummonococcum	A^mA^m
	T. urartu	AA
Tetraploids	*Ae. biuncialis*	UUMM
(2n = 4x =28)	*Ae. gericulata*	MMUU
	(=Ae. ovate)	
	Ae. neglecta	UUMM
	(=Ae. triaristata 4x)	
	Ae. columnaris	UUMM
	Ae. triuncialis	UUCC; CCUU
	Ae. kotschyi	SSUU
	Ae. peregrine	SSUU
	(=Ae. variabilis)	
	Ae. cylindrical	DDCC
	Ae. crassa 4x	DDMM
	Ae. ventricosa	DDNN
	T. turgidum	BBAA
	T. timopheevii	GGAA
	Ae. recta	
Hexaploids	*(=Ae. triaristata 6x)*	
(2n = 6x = 42)	*Ae. vavilovii*	UUMMNN
	Ae. crassa 6x	DDDDMM
	Ae. juvenalis	DDMMUU
	T. aestivum	BBAADD
	T. zhukovskyi	**GGAAA^{m}A^m**

Due to the size and genetic structure of the wheat genome, wheat has been identified as one of the most complex crop species. Brenchley *et al*. (2012) identified between 94000 and 96000 genes across the hexaploid A, B and D genomes by using shotgun sequencing. *T. Aestivum* is hypothesized to be hybridized from a rare cross between a wild tetraploid (AABB) species (2n = 28) and a diploid (DD) species (2n = 2x =14) which are related to *Triticum* and referred to as *Aegilops* L. *Durum* wheat (*T. Turgidum ssp.*) has been nominated as the tetraploid parent and the cytoplasm donor of *T. aestivum* (Feldman *et al.,* 2001 and Matsuoka and Nasuda, 2004). The hybridization events in wheat for example show several species contributing to form the domesticated wheat types. This event made it possible to use related or wild relatives as genetic diversity gene pools for crop improvement (Brozynska *et al*., 2015). The crossability between gene pools of different species and the effort to transfer traits from them is illustrated in Figure 2.1. The effort to transfer traits depends on the flowering synchronization, outcrossing rate, genetic compatibility (ploidy level, genomic similarity and chromosome paring) and the number of progeny produced (Zaharieva and Monneveux, 2006).

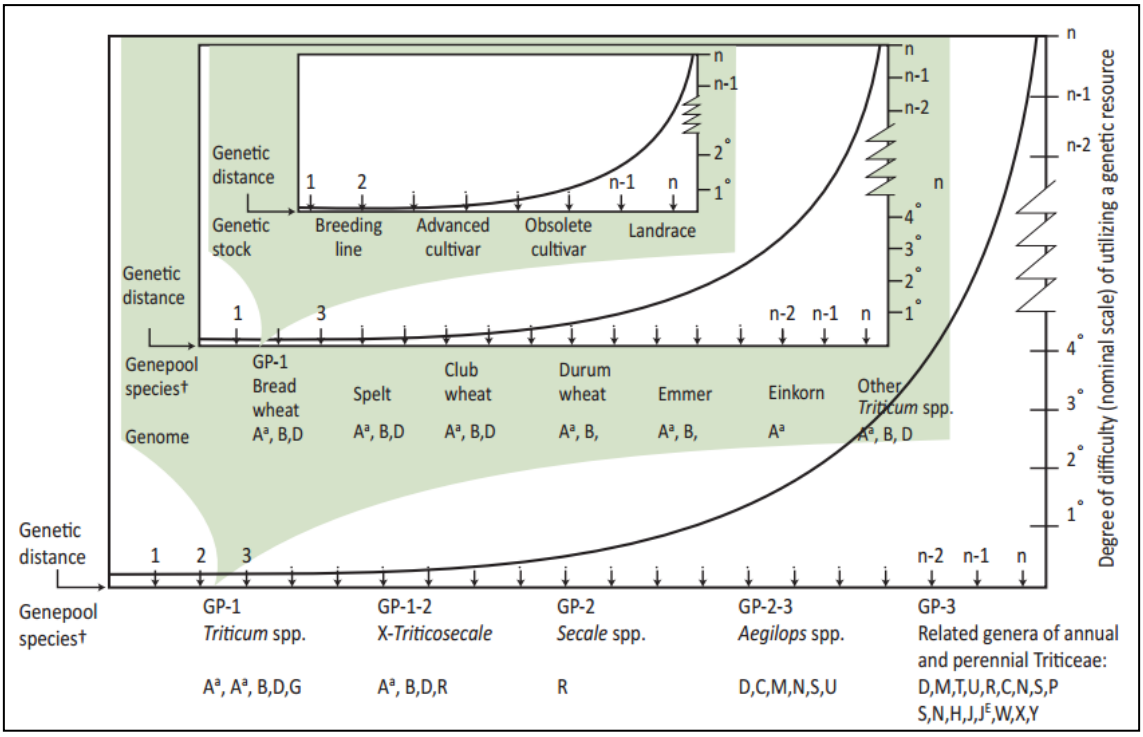


Figure 2.1. The different gene pools and the effort needed to transfer traits from them (Reynolds *et al*., 2012)

The 21 pair homologous chromosomes of bread wheat fall into seven homologous groups. The seven homologous groups form the wheat karyotype, arranged based on the A, B, and D genomes. Each genome contributes one pair of chromosome in each homologous group. Sears (1954) arranged the homologous chromosomes base on their ability to compensate for one another's absence. For example; group 1 will be arranged as chromosomes 1A, 1B, and 1D (Levy and Feldman, 2004). During the domestication process additional quantitative traits were modified such as grain yield, seed size, plant height and heading date. These traits combined with wheat ear number per plant, ear weight per plant, single ear weight and seed number per ear was modified to adapt to specific growing and environmental conditions of other cultivated regions (Kilian *et al.,* 2009). The unfavourable characteristics that were domesticated in wheat are the glumes, brittle rachis, no-free threshability and yield characteristics. Unfortunately the genetic variability of these domesticated wheat types were compromised through the domestication process. Luckily the domestication and land races and traditional varieties were improved to genetically modern cultivars. However this shift has produced a genetic bottleneck causing a decline in diversity in breeding germplasm (Fu and Somers, 2009 and Tanksley and McCouch, 1997). Even though wheat experienced a genetic bottleneck due to inbreeding, wheat still adapts significantly well to abiotic and biotic factors. This is because of the adapted types of wild emmer found in the near Fertile Crescent (Nevo *et al*., 2002). Therefore, the importance of germplasm collection in the agricultural industry is valuable and essential for the conservation of biodiversity (Nevo, 2002; Johnson *et al*., 2003; Nevo and Chen, 2010 and Nevo, 2011).

2.2. Economic importance of wheat

Wheat production started in the 1600s with the arrival of Jan van Riebeeck in 1652 (Du Plessis, 1933). The first wheat breeding programme started in 1891 and through the cultivation and improvements over the years, cultivars of significance have been released. The wheat production regions of South Africa consist of three distinct areas, each with their own challenges and requirements. The Free State Province is typically a dry land production area. The farmers plant winter wheat on stored soil moisture from

the previous rainfall season. The Western Cape Province on the other hand, plant dry land spring wheat in the Mediterranean climates. The third distinct area is where the irrigated spring wheat types are grown and that is next to major rivers in the summer rainfall areas (Van Niekerk, 2001). Together these provinces are responsible for 84 % of the total wheat production (JADAFA, 2014). Wheat in South Africa is the second most important cereal crop being cultivated (JADAFA, 2014). Wheat production however significantly declined from 2001/2002 despite the rising of the wheat prices. The main problem of this trend is the year after year low moisture levels in the summer rainfall areas (BFAP, 2013). Wheat production did increase even though there was a decline in area production, from 2.5 Mt in 2000/2001 to 3 Mt in 2016/2017 (Figure 2.2). This is due to increased technology adaptations and better breeding progresses against abiotic/biotic stressors (SAGIS, 2016).

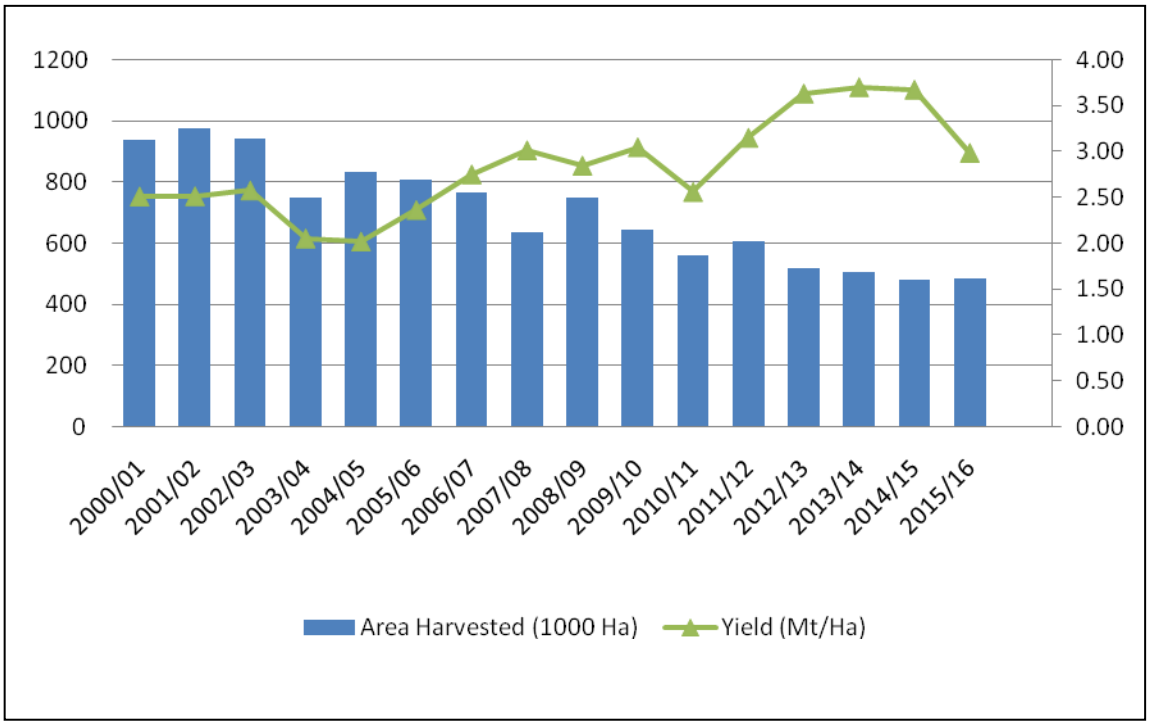


Figure 2.2. Wheat area harvested and yield (2000-2015) (SAGIS, 2016)

2.3. Strategies to accelerate wheat production in South Africa

In the past breeders relied on phenotypic characteristics for the presence of resistant genes. Breeders soon discovered that additional factors influenced the discernment if evaluation was only based on a phenotypic level. These problematic factors were the

complexity during evaluation, low repeatability and the genes of interest being masked by other genes (Kuchel *et al.,* 2007). Although traditional plant breeding contributed significantly during the past decade, major progress has been seen through modern plant breeding with the aid of biotechnology. This progress made it possible for breeders to get a deeper understanding of the trait of interest, not only on a phenotypic level, but also on a molecular level (Breseghello and Coelho, 2013). One benefit molecular breeding adds to a breeding programme, is that the selection process has moved to a genotypic level compared to traditional breeding programs that were only based on a phenotypic level (Ruane and Sonnino, 2007).

Breeding lines have either low agronomic traits or unfavourable genetic backgrounds that are not perfect for the breeding programme. In landraces or wild relatives the genetic variability is mainly hindered by linkage drag or the incompatibility between breeding lines, wild relatives or others. Under these conditions, a pre-breeding programme can help to improve the use of genetic variability in both refined and incompatible wild type germplasm. In a pre-breeding programme desirable agronomic traits/genes from an unfavourable donor germplasm can be identified and transferred to a well-adapted genetic background that can be used directly in a breeding population. With this pre-breeding programme material can thus be obtained that can benefit the plant breeders in their breeding programmes for the development of new varieties with good genetic variability (Figure 2.3) (Sharma *et al*., 2013).

By combining double haploid technology and MAS to the pre-breeding process, the process can be shortened. This helps wheat breeders to develop complete pure homozygous wheat plants in the shortest amount of time. The process involve two important steps the haploid induction and the colchicine treatment for chromosome doubling (Niu *et al*., 2014). MAS help to distinguish the purity, region of interest and genetic diversity of breeding/selecting lines (Collard and Mackill, 2008).

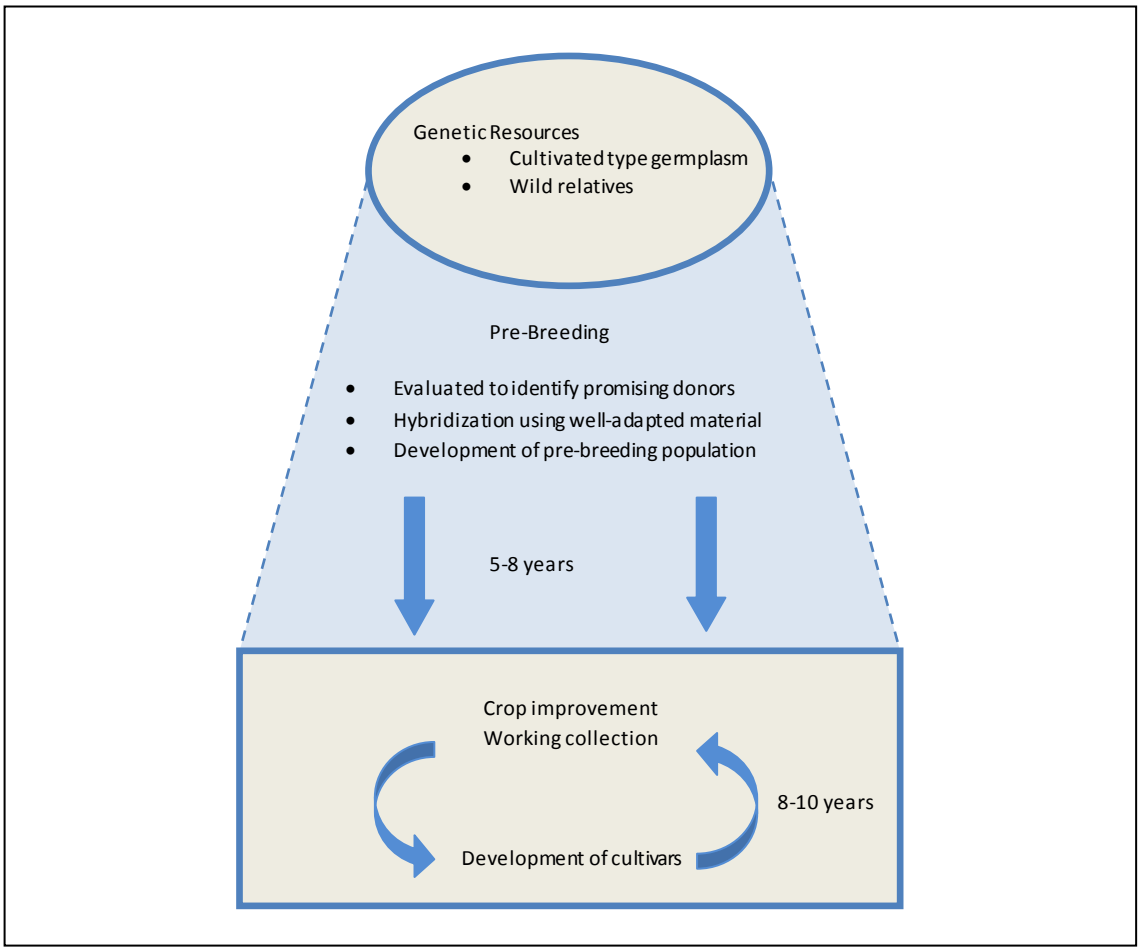


Figure 2.3. Pre-breeding crop improvement scheme (adapted from Sharma, 2013)

2.4. Abiotic and biotic stresses

Wheat cultivation is at times under environmental pressure due to different climate patterns. The changes put strain on breeders to create new wheat cultivars against these devastating abiotic and biotic stressors (Tester and Langridge, 2010). The wheat rusts, *Puccinia* spp., are a significant biotic stressor that continues to threaten sustainable wheat production (Boshoff *et al.,* 2002)

2.5. The wheat rusts

The three wheat rust pathogens of *Puccinia* spp. are adaptable, airborne and can mutate. The rust pathogens travel long distances in the shape of urediniospores (Roelfs, 1988). The pathogens are biotophs which means that the pathogens require a

primary or secondary host to survive and expand (Singh and Roelf, 2002). The sexual and asexual phase of *Puccinia* spp. (Figure 2.4) (Smit, 2013).

Fungicides are used as the primary control mechanism against these diseases, but through resistant breeding, cultivars can be developed to reduce the amount of fungicides being sprayed (Smale *et al*., 1998). The continued adaptation of the fungi and its ability to travel long distances are the two main reasons why breeders continue to search for new resistance sources. Resistant genes from wild *Triticeae* species can be transferred into wheat species to increase the number of available resistant genes. Unfortunately single resistant genes have a short longevity that is easily overcome by the wheat rust fungi. Combining several rust resistance genes is a more durable form of resistance compared to single genes (Kolmer, 2005).

2.5.1. Stripe Rust

Stripe rust also referred to as Yellow rust is caused by *Puccinia striiformis* f. sp. *tritici* it is known to infect wheat, but is not limited too as infection was reported in barley, rye and triticale. The United States was the first country to report stripe rust (Carleton, 1915). It was soon followed by other countries in East Asia, South Asia, Australia, New Zealand, East Africa, Yemen and Western Europe (Wellings, 2011). Stripe rust infects leaves, the head, and sheaths and rarely stems. Symptoms of stripe rust are chlorotic lesions on the leaves of the primary host (wheat). Inside these lesions yellow-orange uredinia develop. The uredinia develop inside along the leaf veins into what we see as stripes on the leaf blades (Figure 2.5). During the growing season these uredinia produces urediniospores (20µm to 30 µm in diameter) the spores are dispersed by wind and rain.

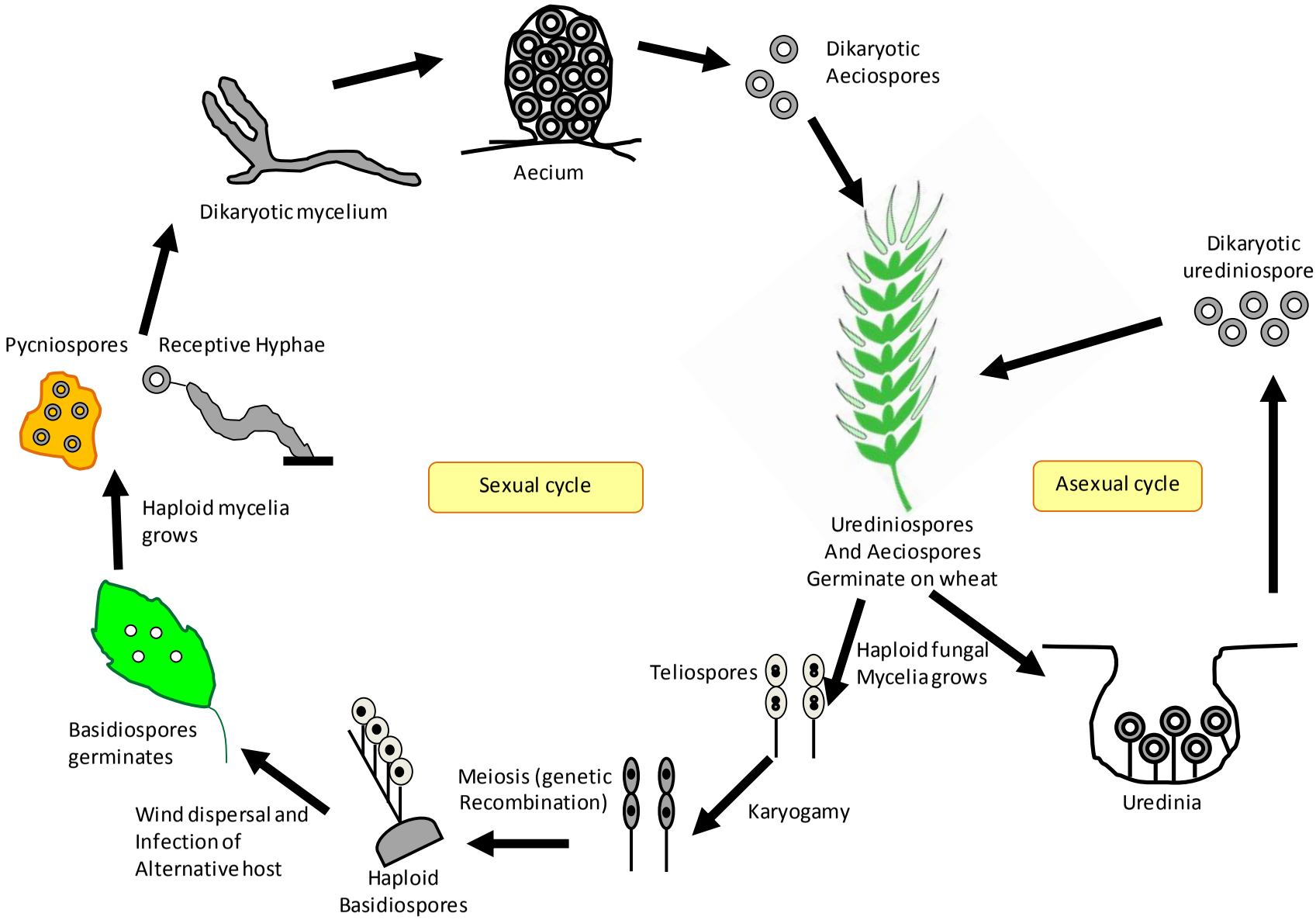


Figure 2.4. The asexual and sexual phase of the *Puccinia* spp. life cycle (adapted from Smit, 2013)

Stripe rust can overwinter; the lesions change to dark brown telia which are produced in the epidermis of leaves and release teliospores in the following growing season. The fungus overwinters as mycelium or urediniospores (Zadoks, 1961). Stripe rust differ from leaf and stem rust, by thriving in low temperatures and higher altitudes. The optimum temperature is between 10°C and 15°C. This limits the pathogens ability to cause major damage in wheat regions with a higher optimum temperature. However, adaptability of stripe rust has been reported in warmer climates, due to the change in climate patterns (Hovmoller *et al.,* 2011). The sporulation aggressiveness of *P. Striiformis* was also reported by Milus *et al*. (2009) at temperatures exceeding the optimum threshold of *P. Striiformis*.

The first stripe rust report in South Africa was from the Western Cape Province in 1996 (Pretorius *et al*., 1997). The race 6E16A^{-} showed strong similarities with pathotypes from East and North Africa, southern Europe, Western Asia and Middle East (Enjalbert *et al*., 2005). Ali *et al*. (2014) confirmed the origin of the South African race 6E16A^{-} to be from the Mediterranean/Central Asia countries. Spread was soon reported in wheat production areas after the first report in 1996 (Boshoff *et al*., 2002). Mutational events also occurred in the four races that were reported (Pretorius *et al*., 2007; Agenbag *et al*., 2012).

Figure 2.5. Stripe rust on wheat (Photos: left: W.C. Botes and right: Z.A. Pretorius)

It is very important to monitor and genotype the *P. Striiformis* races found in South African through SSR analysis regularly (Pretorius *et al*., 2016). The four races with the year of identification and their Avirulence/virulence genes are listed in table 2.2 adapted from a study done by Pretorius *et al*. (2016).

Table 2.2. The four races with the year of identification and their Avirulence/virulence genes

Race	Year reported	Avirulence/virulence	References
6E16A-	1996	*Yr1, Yr3a, Yr4a, Yr5, Yr9, Yr10, Yr15, Yr25, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* / *Yr2, Yr6, Yr7, Yr8, Yr11, Yr14, Yr17, Yr19*	Pretorius *et al*. (1997)
6E22A(+)	1998	*Yr1, Yr3a, Yr4a, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* / *Yr2, Yr6, Yr7, Yr8, Yr11, Yr14, Yr17, Yr19, Yr25*, (*YrA*)	Boshoff and Pretorius (1999)
7E22A-	2001	*Yr3a, Yr4a, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* / *Yr1, Yr2, Yr6, Yr7, Yr8, Yr11, Yr14, Yr17, Yr19 Yr25*	Pretorius *et al*.(2007)
6E22A+	2005	*Yr1, Yr3a, Yr4a, Yr5, Yr9, Yr10, Yr15, Yr27, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* / *Yr2, Yr6, Yr7, Yr8, Yr11, Yr14, Yr17, Yr19, Yr25,*(*YrA*)	Pretorius *et al*. (2016)

2.5.2. Leaf rust

Leaf rust (*Lr*) caused by *Puccinia triticina* Eriks is one of the most important diseases on wheat (Curtis *et al*., 2002). Leaf rust causes significant yield losses by reducing floral set and causes grain to shrivel. These losses are severe during the summer season when the dew period is longer than 3 hours and the temperature is ≥ 20°C. The longer the dew period and temperature stays favourable the higher the infection. Few or any infection occurs when temperatures exceeds 32°C or below 2°C (Stubb *et al*., 1986). Leaf rust also cause losses when latent infections over-winter on wheat crops or alternative hosts. Losses due to leaf rust are low or equal to 10 %, but 30 % losses have previously been reported (Todorovska *et al*., 2009). *P. triticinia* starts by developing orange brown circular uredinia on the top and bottom part of the wheat leaves (Figure 2.6). Brown spores are being produced by the uredinia, the spores are 20 µm in diameter. The symptoms are nectrotic lesions that develop on the host leaves. Shortly

after the development of the nectrotic lesions, teliospores can germinate when moisture are available to produce basidospores which can infect the alternative host assuring the survival of yet another leaf rust cycle. The sexual recombination cycle starts when the basidospores infect the alternative host. The basidospores develop aeciospores which will again infect the primary host. Urediniospores are released from the uredinia which will infect the host plant once optimum conditions are favourable (Bolton *et al*., 2008).

More recent Terefe *et al*. (2014a, b) reported three new *P. triticina* races present in South Africa. The new races were coded as CCPS, MCDS and FBPT it is strongly hypothetically introduced from neighboring countries (Terefe *et al*., 2014a, b). This statement is supported by Pretorius *et al*. (2012) whom reported lineages of wheat stem rust found in South Africa and Zimbabwe. Based on a study done in 2015 by Pretorius *et al*. (2015) four new *P. triticina* lineages was identified in neighboring countries (Zimbabwe, Zambia and Malawi). Table2.3 list the new races found in South African neighbouring countries and their avirulence/virulence genes. These new *P. triticinia* races can be linked to inoculums exchange in the country and can be speculated as the reason for new races in South Africa (Pretorius *et al*., 2015).

Figure 2.6. Leaf rust on wheat (Photo: W.C. Botes)

Table 2.3. The new races found in South African neighbouring countries and their avirulence/virulence genes (Pretorius *et al*., 2015)

Year	Country	Race	Avirulence/virulence
2011	Zimbabwe	MCDS	*Lr2a, 2c, 3ka, 9, 11, 16, 18, 24, 30 / 1, 3a, 10, 14a, 17, 26, B*
	Zimbabwe	TCPS	*Lr9, 11, 16, 18, 24 / 1, 2a, 2c, 3a, 3ka, 10, 14a, 17, 26, 30, B*
2012	Zimbabwe	MCDS	As above
	Zimbabwe	FBPT	*Lr1, 2a, 9, 11, 16, 24, 26 / 2c, 3, 3ka, 10, 14a, 17, 18, 30, B*
	Zambia	MCDS	As above
	Zambia	TCPS	As above
2013	Malawi	SCDS	*Lr3a, 3ka, 9, 11, 16, 18, 24, 30 / 1, 2a, 2c, 10, 14a, 17, 26, B*

2.5.3. Stem rust

Stem rust or black rust is caused by *P. graminis* f. sp. *tritici* (Todorovska *et al*., 2009). The symptoms of stem rust are mostly found on stems and sheaths. The first sign of infection is a small necrotic lesion about 8-10 days after infection (Figure 2.7) (Leonard and Szabo, 2005). A new stem rust linage 2SA88 (TTKSF) that belong to the Ug99 group have been identified. What made this linage so important was the wide virulence spectrum. After the discovery of this new linage, it was found that the single mutation that occurred in this linage resulted in virulence against the *Sr24* resistance gene in the Western Cape. This finding triggered the problem of single resistance in new cultivars.

Figure 2.7. Stem rust on wheat (Photo: W.C. Botes)

Stem rust was feared in many countries where wheat is cultivated. The fear was driven by the ability of stem rust to infect a healthy cultivar in 3 week before harvest, ending with black broken stems and shrivelled grains (Singh *et al*., 2006). Stem rust can cause significant losses of up to 50 % when conditions are favourable. Since the first epidemic report of stem rust in South Africa in 1926, Pretorius *et al*. (2007) identified 16 different stem rust lineages. In 1998 the new stem rust race Ug99 or TTKSK was identified in Uganda and showed virulence to *Sr31* (Pretorius *et al*., 2000) and *Sr38* genes (singh *et al.,* 2006). Ug99 is changing and spreading very quickly in Africa. The most recent country being affected in Africa with Ug99 lineages are Egypt, Ethiopia, Kenya, Mozambique, Rwanda, South Africa, Sudan, Tanzania, Uganda and Zimbabwe (Pretorius *et al*., 2000, 2010, 2012; Boshoff *et al*., 2002;Jin *et al*., 2008, 2009; Nazari *et al*., 2009;Pretorius *et al*., 2010; Mukoyi *et al*., 2011 and Wolday *et al*., 2015). Recently a study done by Terefe *et al*. (2016) reported three new races BFBSC (2SA108.1, 2SA108.2 and 2SA108.3) and via cluster analysis the genetic distinctness was expected. The race was identified from a 2010 Greytown rust nursery. The new race is not related to the Ug99lineages, but it is similar to other South African lineages. Table 2.4 list the avirulence/virulence profile of the BFBSC race and the other known races identified in the study (Terefe *et al*., 2016).

Table 2.4. The avirulence/virulence profile of the BFBSC race and the other known races (Terefe *et al*., 2016)

Race	Avirulence/virulence profile
TTKSF	*Sr9h, 24, 27, 31, 36, Kiewiet, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 21, 30, 38, McN*
TTKSF + *Sr9h*	*Sr24, 27, 31, 36, Kiewiet, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 9h, 10, 11, 17, 21, 30, 38, McN*
BPGSC + *Sr27*	*Sr5, 6, 7b, 8b, 9e, 17, 21, 24, 30, 31, 36, 38, Kiewiet, Satu, Tmp/8a, 9a, 9b, 9d, 9g, 10, 11, 27, McN*
BNGSC + *Sr27*	*Sr5, 6, 7b, 8b, 9e, 9g, 17, 21, 24, 30, 31, 36, 38, Kiewiet,Satu, Tmp/8a, 9a, 9b, 9d, 10, 11, 27, McN*
BPGSC + *Sr27*,*SrKiewiet*	*Sr5, 6, 7b, 8b, 9e, 17, 21, 24, 30, 31, 36, 38, Satu, Tmp/8a, 9a, 9b, 9d, 9g, 10, 11, 27, Kiewiet, McN*
BPGSC + *Sr27*, *SrKiewiet*, *SrSatu*	*Sr5, 6, 7b, 8b, 9e, 17, 21, 24, 30, 31, 36, 38, Tmp/8a, 9a, 9b, 9d, 9g, 10, 11, 27, Kiewiet, Satu, McN*
TTKSP	*Sr9h, 27, 31, 36, Kiewiet, Satu, Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 21, 24, 30, 38, McN*
PTKST	*Sr9h, 21, 27, 36, Kiewiet, Satu,Tmp/5, 6, 7b, 8a, 8b, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 24, 30, 31, 38, McN*
BFBSC	*Sr5, 6, 7b, 8b, 9b, 9e, 11, 17, 21, 24, 30, 31, 36, 38, Kiewiet, Satu, Tmp/8a, 9a, 9d, 9g, 9h, 10, 27, McN*

2.6. Resistance systems

Rust resistance are governed through two categories: (A) race-specific resistance (hypersensitive gene for gene response) and (B) Adult plant resistance (APR) (quantitative resistance) often conferred by multiple loci, and is not associated with a gene for gene hypersensitive response (HR). The resistance durability significantly favours Adult plant resistance when compared with race-specific resistance however; it must be improved over multiple cycles (Parlevliet, 2002).

Resistant cultivars have been the best integrated approach for resistance against cereal rusts (Johnson, 1981). Cultivars like 'Thatcher' and 'Hope' maintained significant stem rust resistance for many years (Hare and McIntosh, 1979). Leaf rust cultivars that stood their ground are'Americano 25', 'Americano 44d', 'Surpreza', 'Frontana' and 'Fronteira' (Perez and Roelfs, 1989 and Roelfs, 1988). Stripe rust cultivars that stood their ground are'Wilhelmina', 'Capelle-Desprez', 'Manella', 'Juliana' and 'Carstens VI'. The average lifespan of a successful cultivar is five years or more (Stubbs, 1985).

The most frequently used rust gene is the *Sr2* adult plant resistance gene. Breeding companies have incorporated *Sr2*on a global scale since 1940s. The wheat-rye translocation (1BL.1RS) associated with *Sr31*, *Lr26* and *Yr9* provided a highly/moderate resistance worldwide to stem rust, but unfortunately the effectiveness only lasted until 1998 in Uganda (Pretourius *et al*., 2000). Approximately fifty resistance genes linked to rust have been catalogued; unfortunately the majority are virulent. Therefore, a combination of these race-specific genes will be the best control strategy for durable resistance (Khan *et al.,* 2005; Singh *et al.,* 2006).

2.6.1. Race specific resistance

Race specific resistance has traditionally been built on a gene-for-gene interaction. Most of the stem rust resistance genes are built on this concept. A gene for gene interaction is where a single resistance gene (R) in the host, recognises a single corresponding elicitor molecule produced by the pathogen (Jones, 2006). Upon recognition the host plant activates the resistance R gene and stops the infection process of the pathogen. This normally results in a hypersensitive response (HR) being

activated within the host cell. Causing adjacent cell death, this response stop the infection within the host cell, making the host avirulent (Jones, 2006).

Resistance based on race specific resistance have been successfully implemented in breeding programmes for crop protection. However the disadvantage of this approach is that the pathogen can overcome the race specific resistance. The pathogen can either mutate or cause deletions during the segregation process. The end result is virulent stem rust lineages. Therefore multiple race specific resistance R genes are needed to be incorporated into a cultivar for sustainable resistance. Breeders either search for new R genes from unimproved germplasm of the same species or from related species that are compatible (Steffenson, 1992).

Race specific resistance has a short life expectancy, due to the mutating ability of the pathogen. There are however several single resistance genes that stud their ground against rust pathogens for 50 years (Steffenson, 1992). One example is the *Rpg1* stem rust gene, it encode a serine-threonine protein kinase that degrade the infected tissue through the proteasome when the effector gene of the pathogen is being recognized (Brueggemann *et al*., 2002; Nirmala *et al*., 2006 and Nirmala *et al*., 2007). Other R-genes is *Sr26* and *Sr31*. The*Sr31* gene provided significant resistance in India, China, Europe and South America until the new Ug99 stem rust lineage that evolved in Uganda in 1998 (Mago *et al*., 2004, 2005). Therefore to maintain elite cultivars a combination of multiple R-genes needs to be combined to insure durable resistance are being incorporated in new wheat cultivars being released (Singh *et al*., 2006).

2.6.2. Adult plant resistance

Another class of resistance is horizontal resistance also referred to as APR or slow rusting resistance (Caldwell, 2005). Adult plant resistance is more abundant in adult plants when compared to seedlings but, not all APR are quantitative resistance. There are some single race-specific R-genes for example *Lr34* that are associated with APR. Advantages of APR; it provide resistance against all isolates. Secondly APR is much more durable compared to R-genes. Breeding for APR can be challenging and can take a lot of time. Some challenges that breeders face when breeding for APR is; genotypes do not have all the minor genes that are of interest. The minor genes can be lost due

to segregation, leading to minor gene losses. The genotype can't be used directly as a breeding source. Breeding programmes can be more adapted to select major genes. Molecular markers can mask the gene of interest. The cost of utilizing multiple markers can be expensive (Singh *et al*., 2011).

Successful examples of resistance based on minor genes are the slow rusting resistance against leaf and stripe rust in many CIMMYT wheat lines. The concept of Caldwell (1968) was adapted based on slow rusting resistance in selecting segregating populations that showed 20-30 % of severity compared to susceptible wheat plants. This concept leads to the release of 'Nacozari 76', 'Pavon 76', 'Tarachi 2000' and 'Rayon 89' (Singh *et al*., 2011).

2.7. Wheat rust resistance genes important for the current study

2.7.1. *Lr62* and *Yr 42*

Lr62 and *Yr42* were translocated to hexaploid wheat from the goat grass family *Aegilops neglecta* (Marais *et al*., 2009). The genes are located on chromosome 6AS.The original translocation of *Ae. Neglecta* favoured female transmission in comparison to the other resistance genes that favour male pollen transmission. The challenge however was to reduce the amount of unwanted DNA. Marais *et al*. (2010b) was successful in his attempt to reduce and replace the unwanted DNA translocation with wheat chromatin. The recombination was achieved by the absence of the *Ph1*. *Ph1*restrict the paring process of the homologous regions. With the absence of *Ph1*paring between the telomere regions will be possible (Marais *et al.,* 2010b). The markers used by Marais *et al*. (2010b) were *Xgwm427*, *Xbrac171* and a SCAR marked *Xsopw7*. The markers were used to perform a tree-point test cross analysis to create a small exclusive recombinant group of plants associated with *Lr62*. The selected recombinant lines were additionally screened with markers (*Xcfd190*, *Xcfa2173*, *Xgwm334*, *Xwmc256* and *Xgwm169*).

2.7.2. *Lr59*

The gene *Lr59* on chromosome 1AL was carried over to hexaploid bread wheat from a goat grass species *Aegilops peregrine* (Marais *et al*., 2008). Marais *et al*. (2008) performed a backcross witha line from Israel carrying *Lr59* to hexaploid bread wheat. The backcross causes a spontaneous resistance event; later the cause was established to be a centromeric break and fusion event when the *Lr59* was transferred (Marais *et al.,* 2008). In 2009 Kotze successfully reduced the amount of *Ae. Peregrina* present in the wheat lines. Researchers argued that the S-genome is the donor genome for *Lr59* due to the wheat like similarity (Kotze, 2009). The markers used by Marais *et al*. (2008) were *Xgwm136*, *Xcfa2153-1A*, *Xbarc17-1A*, *Xbarc83-1A*, *Xgwm99-1A*, *Xcfa2135-1A*, *Xbarc187-1B*, *Xgwm550-1B*, *Xgwm413-1B*, *Xgwm337-1D*, *Xgwm458-1D* and *Xcfd65-1D*.

2.7.3. *Lr53* and *Yr35*

The*Lr53* and *Yr35* are two seedling resistance genes that were transferred from *T. dicoccoides* to wheat. Marais *et al*. (2005b) also transferred these seedling resistance genes to hexaploid bread wheat. *Lr53* and *Yr35* are found to be located on chromosome 6BS. Reports of virulence have not yet been reported giving these two resistance genes importance for upcoming resistance breeding programmes against rust pathogens. Recombination between the two genes was identified (11 %) by Marais *et al*. (2005b) and later (3 %) by Dadkhodale *et al*. (2011). This indicated that different recombinations can occur between the two genes depending on the genetic background (Marais *et al*., 2005b). The closest marker linked to *Lr53* was *Xcfd1* with a distance of 1.1cM. The closest marker to the *Yr35* gene was *Xgwm191* with a distance of 18.9 cM (Dadkhodale *et al*., 2011).

2.7.4. *Lr56* and *Yr38*

The *Lr56* and *Yr38* genes were transferred from *Aegilops sharonensis*. These genes are located on chromosome 6AL (Marais *et al*., 2006). The translocation chromosome is almost entirely from *Aegilops sharonensis* with only the distal end being from the wheat chromosome 6AL. This resulted in problems when lines were crossed to transfer genes. The result is a lot of unwanted foreign DNA which can be undesirable for

breeding purposes. Marais *et al*. (2010a) did however remove large amount of foreign DNA also through homoeologous recombination without losing any valuable agronomical traits. The markers used to identify recombinant plants were (*Xgwm427*, *Xbarc171*, *Xcfd19*0 and *Xgwm334*). The selected recombinant plants were additionally screened with 6AL microsatellites (*Xgwm617*, *Xgwm570*, *Xwmc256*, and 6AS microsatellites *Xgpw2295*and *Xgwm459*). The findings stated that most of the 6AL and *Lr56/Yr38* translocation are homoeologous (Marais *et al*., 2010a).

2.7.5. *Lr54* and *Yr37*

Leaf rust *Lr54* and yellow rust *Yr37* was transferred from *Aegilops kotschyi*.It is a wild tetraploid grass species with a UUSS genome. The translocation was originally from a double monosomic 2D plant and an unknown *Ae. Kotschyi* group 2 chromosome.*Lr54* is effective against pathotypes of South Africa (Marais *et al*., 2003). Marais *et al*. (2005a) hypothesised that the translocation of *Lr54* and *Yr37* could have occurred through arbitrary fusion. Chromosomes that are unpaired cause a misdivision at cell division. The end result is a breakage at the centromeres, causing telocentric chromosome development that is revered to as arbitrary fusion (Marais *et al*., 2003). Homologous recombination was prevented due to the structural discrepancies between the 2DL wheat chromosome and the chromosome arm of *Ae. Kotschyi*. Genetic maps that are available provide significant markers that are evenly distributed across the region. Markers that were mapped to chromosome 2DS was *Xgwm261,* X*gwm484* and *Xcfd116* (Heyns *et al*., 2011).

2.7.6. *Sr24*

Sr24 is avirulent to Ug99 and most lineages of stem rust except for the most recent lineage TTKST. Smith *et al*. (1968) determined the location of *Sr24* to be on chromosome 3DL. The discovery was determined through a spontaneous translocation of *Agropyron elongatum*. Sears, (1973) however introduced more genetically modified lines and successfully determined a much smaller *A. elongatum* translocation of *Sr24*. Later it was discovered that the leaf rust resistance gene *Lr24* is also linked to all of the translocations associated with *Sr24*. Several molecular markers exist for *Sr24*. It

includes an SSR and two AFLPs. For the SSR marker it is BARC71 and for the AFLPs it is *Sr24#12* which are completely linked to *Sr2*. The other AFLP is *Sr24#50* which do not predict the presence of *SR24* in some germplasm.

2.7.7. *Lr34*

The *Lr34*gene was first described in 'Frontana' in 1966. *Lr34* is one of the many slow rusting genes, which provide durable resistance. It is located on chromosome 7SD. When the tips of the leaves of the wheat plant turn necrotic it is a positive morphological characteristic of the presence of *Lr34*. This necrotic trait of *Lr34* is due to the association *Lr34* has with the leaf tip necrosis (LTN) locus. *Lr34* also provide resistance to Powdery mildew, leaf tip necrosis and barley yellow dwarf virus. The effectiveness of *Lr34* gene has been for more than 100 years (Keller *et al*., 2013). However, durable resistance will only be possible if *Lr34* is used in combination with other major/minor resistance genes (Krattinger *et al*., 2009). A study done by Singh and Huerta-Espino (1995) reported that a 40 % disease severity was observed in cultivars with only *Lr34*, but in combination with one or two minor genes a disease severity of only 10-15 % was observed. *Lr34* is also associated with an unknown accumulation of an electro dense substance. The substance causes a thickening of the mesophyll cell wall. The thickening reduces haustoria formation of the fungi.

Bossolini and co-workers and Lagudah and co-workers developed the first markers (Swm10) and (csLV34) linked to *Lr34* in 2006 respectively. Later Lagudah and co-workers (2006) developed markers cssfr1 to cssfr7 due to limiting factors of markers SWM10 and csLV34.

2.7.8. *Sr26*

Sr26 was transferred from a foreign segment of *Agropyron elongatum* (syn. *Thinopyrum ponticum*) to hexaploid wheat. *Sr26* is a resistance gene against Ug99 and its lineage TTKSK. It is located on chromosome 6AL (Dundas *et al*., 2007). *Sr26* haven't been used as a resistance source based on recent screens of 170 lines around the world. This was because *Sr26* was only used as a resistance source in Australian cultivars such as Eagle, released in 1971. After the 9% yield reduction in Australian

wheat cultivars, the use of *Sr26* has come very limited. The effectiveness of *Sr26* makes this a favourable resistance gene for gene pyramiding. It's favourable for the following: (A) resistance against the Ug99 and its lineages; (B) low frequency among modern cultivars and (C) good agronomical refined donor lines (Liu *et al.*, 2010). With marker assisted selection a combination of two dominant SSR markers can be used for the presence of *Sr26 (*Sr26#43) and a marker for the absence of *Sr26* (Liu *et al*., 2009; Mago *et al*., 2005).

2.7.9. *Lr19*

The *Lr19* gene was introduced from *Thinopyrum* spp. to the cultivar 'Argusand' and is located on chromosome 7D (Autrique *et al*., 1995). The first virulence in *P. triticinia* was reported by Singh *et al*. (2000).*Lr19* and *Sr25*are linked to the same translocation (Prins *et al*., 2001). *Lr19*is not favoured by breeders. A yellow pigment linked to a *Y* gene found in the original translocation is the negative effect of *Lr19*. For bread making properties in many countries it is an undesirable characteristic, but then again favourable for pasta making (Zhang *et al*., 2005). The linkage between *Lr19* was broken through allosynthetic paring and crossover in 'Indis'. The recombinant line showed no yellow endosperm and was located on the 7BL chromosome (Prins *et al*., 1997). The marker linked to *Lr19* is a dominant STS marker ($STSLr19_{130}$). This marker is distant from *Lr19* (Prins *et al*., 2001).

2.7.10. *Sr2*

Sr2 is a non-specific recessive gene. *Sr2* is known for its slow rusting response in wheat (Singh *et al*., 2002) and is one of the most successful adult plant resistance (APR) genes against stem rust since 1920 (Kota *et al*., 2006). It was transferred to 'Hope' from 'Yaroslav' emmer wheat by McFadden in the US in 1930.*Sr2* is located on chromosome 3B and provides partial resistance to all of the stem rust races. The *Sr2* gene is however not sufficient against Ug99 on its own (Singh *et al*., 2006). When combined with minor genes, the resistance response against stem rust is effective and durable (Rajaram *et al*., 1988 and Shingh *et al*., 2006). *Sr2* can be identified through a specific phenotypic characteristic known as pseudo-black chaff (PBC). PBC is closely linked to *Sr2*, although

too much expression of PBC can however be an undesirable trait. Another closely linked minor gene to *Sr2* is the APR *Yr30* gene that confer slow rusting resistance against stripe rust (Knott, 1982 and 1988).

Traditionally the presence of PBC was the selection mean for *Sr2* resistance. With marker technology the presence of*Sr2*can be detected much more accurately. Different microsatellite markers have been designed to help with the identification of this *Sr2* gene. The best marker today is the*csSr2*marker. This marker was developed in 2013 by Mago *et al*. (2011). The *csSr2* was derived from three different alleles of *Sr2*; the 'null' allele, the 'Marquis' type allele and the 'Hope' type allele. The 'hope' type allele is the allele associated with *Sr2*. By cutting the fragment with (*Bsp*HI), three different bands with three different sizes (172 bp, 112 bp and 53 bp) should be present to be positive for the presence of *Sr2*.

2.8. Fusarium head blight

Fusarium head blight (FHB) is a highly destructive wheat disease that causes significant yield and quality losses. The causal pathogen, *Fusarium graminearum* can cause the accumulation of harmful mycotoxins in the grain. Mycotoxins are low molecular weight metabolites produced by fungi (Desjardins and Hohn, 1997). Toxins are chemically and thermally stable and can be found in raw grain and finished products. The most common mycotoxins are deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), nivalenol (NIV) and its acetylated derivative 4-acetyl-nivalenol (4ANIV) (Desjardins *et al*., 2006).

Masked mycotoxins are also produced by *Fusarium* species. Although not routinely analysed. Masked mycotoxin or conjugated mycotoxins can cause a health hazard, if toxins are released during digestion (Rychlik *et al*., 2014). A number of conjugated forms of DON, zearalenone and fumonisins have been found and studied by Berthiller *et al*. (2013). No threshold level for masked DON has been set by the European Commission. This can lead to a health hazard if DON-3-glucoside can pass the radar

undetected and cause serious health consequences for human and animals (Panel, 2014).

FHB may be caused by several *Fusarium* species, it includes *F. avenaceum* (Fries) saccardo, *F. crookwellense, F. graminearum, F. culmorum, F. langsethiae* Torp and Nirenberg, *F. poae*(Peck) Wollenweber, *F. Sporotrichioides* Sherbakoff, *Microdochiumnivale* (Fr.) Samuels and I.C. Hallett (Dill-Macky, 2010). FHB ooccurs in a number of regions (North and South America (Walker *et al.*, 2001), Europe (Yli-Mattila *et al.*, 2009), Australia (McKnight and Hart, 1966), Canada (Fernando *et al.*, 2006), Kenya (Boutigny, 2011) and South Africa (Van Wyk *et al*., 1988). In South Africa FHB is a significant concern, because wheat is extensively being used as a staple food and feed (Marasas *et al*., 1977). Because of the significant amount of people and animals that uses wheat in their diet. The need to produce FHB resistant cultivars with reduced mycotoxin levels have become even greater (Bai and Shaner, 2004).

Several outbreaks occurred in the 1900s in North America with losses of up to $4.8 billion. Since this devastating occurrence intensive breeding programmes have been launched since 1997. The challenge however is still to pyramide these resistant QTL in one cultivar, making QTL pyramiding still the best breeding concept for stable and durable resistance (Johnson *et al*., 2003 and Windels, 2000).

2.8.1. Life cycle

The survival of *Fusarium graminearum* is significant, making the control of *Fusarium graminearum* even more difficult. FHB can survive on crop debris from a previous season or on weeds, other plants, seeds and insects (Parry *et al*., 1995; Champeil *et al*., 2004). FHB is predominantly being caused by ascospores from the sexual stage and macroconidia from the anamorph stage (Figure 2.8) (Parry *et al*., 1995; Bai and Shaner, 1994 and Sutton, 1982). The pathogen can survive as a saprophyte on dead host plants. Infection of the wheat spikes are caused through water splash or wind containing ascospore or macroconidia. In warm temperature and high humidity ascospore are prone to germination forming perithecia. From 16°C a perithecia can form and as the temperature increases to about 25°C, the amount of perithecia also increase, resulting in even more infecting of wheat heads.

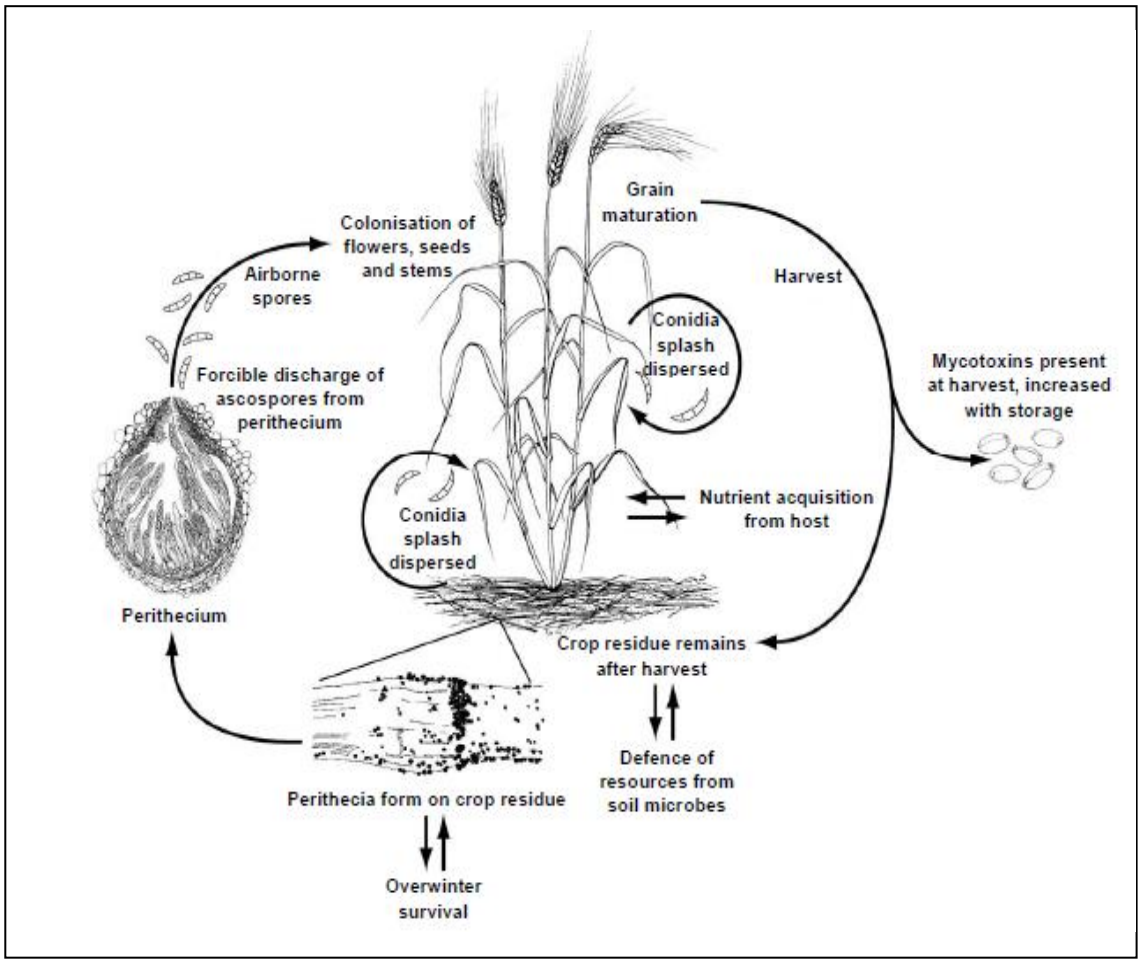


Figure 2.8. The Life cycle of Fusarium head blight (Trail, 2009)

2.8.2. Occurrence

FHB is a global disease that occurs in a number of wheat growing regions (Asia, Australia, Canada, Kenya, North and South America, Europe and South Africa) (Waalwjik *et al.*, 2003; Guo *et al.*, 2008; Karugia *et al*. 2009). The first FHB report on wheat in South Africa was in 1980 in the North-West Province (Scott *et al.*, 1988). Only six of the *Fusarium* species were found in South Africa (Figure 2.9). These species in clued: *Fusarium graminearum, Fusarium meridionale, Fusarium boothii, Fusarium acaciae-mearnsii, Fusarium cortaderiae and Fusarium brasilicum*(Table 2.5). *F. graminearum* was the dominant pathogen in all of the 9 provinces of South Africa (Boutigny *et al*., 2011).

Table 2.5. The six Fusarium species found in South Africa(adapted from Boutigny *et al*., 2011)

Provinces	*F. graminearum*	*F. meridionale*	*F. boothii*	*F. cortaderiae*	*F. brasilicum*	*F. acacia-mearnsii*	*N*
Western Cape	75	0	0	25	0	0	8
North west	100	0	0	0	0	0	12
Limpopo	100	0	0	0	0	0	16
Free State	50	4	46	0	0	0	26
Mpumalanga	88	6	6	6	0	0	34
KwaZulu-Natal	78.7	9.3	5.3	1.3	0	5.3	75
Northern cape	94.3	0	4.7	0	0.9	0	106

Wheat breeders worldwide are trying to contain FHB through resistance breeding strategies. Breeding for resistance with good quality and yield is extremely difficult due to environmental differences of each wheat growing area. Resistance is affected by environmental conditions, which makes it difficult to score disease severity. Wheat varieties are in favour of infection at the flowering stage and grain fill (Dill-Macky, 2010).

2.8.3. Resistant types

Fusarium head blight resistance is expressed by quantitative gene (Löeffler *et al*., 2009; Buerstmayr *et al*., 2009 and Liu *et al*., 2009). It is classified into two resistant mechanisms, morphological and physiological (Gilsinger *et al*., 2005). Physiological resistant are the types we are interested to FHB in wheat. Type I and II, Type I resistance is the first hurdle the host express to infection by Fusarium species. It is complete resistance to infection and is measured as disease incidence and disease severity. Type I resistant quantitative trait loci (QTLs) in the FHB-resistant germplasm 'Wangshuibai', include *Qfhi.nau-5A* on chromosome 5A and *Qfhi.nau-4B* on chromosome 4B.

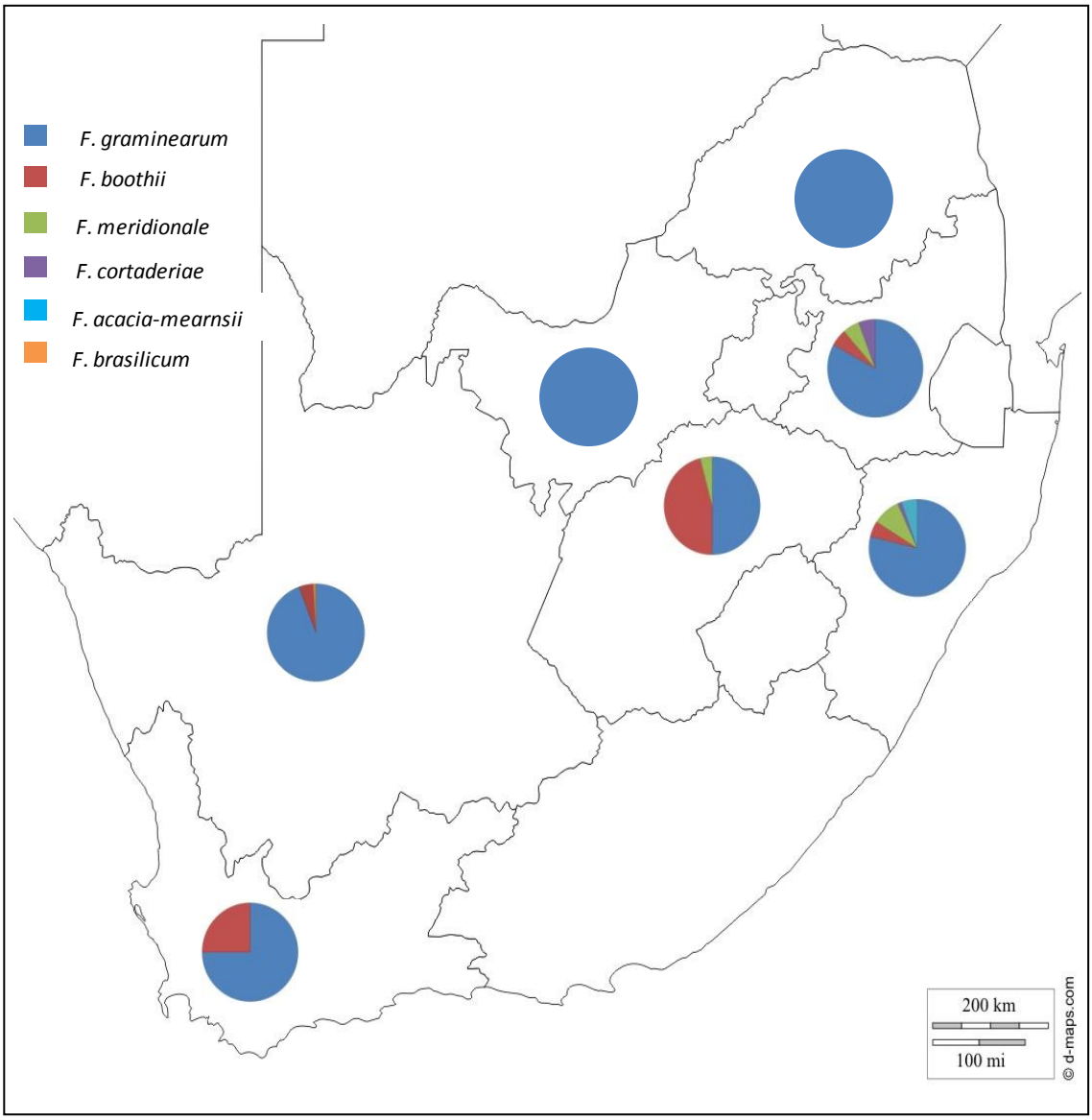


Figure 2.9. Geographical distribution of *Fusarium graminearum* according to wheat host in South Africa (adapted from Boutigny *et al*., 2011)

Type I resistance are classified under morphological variation as it indicated more primary differences in initial infection. Type II resistance is the second hurdle against fungal spread from infection point within spikes and have been reported in 'Sumai 3' (Browne, 2009). The QTLs responsible for type II resistance on chromosomes 3BS and 6BL in 'Sumai 3' have been characterised and mapped as *Fhb1* and *Fhb2.* Type II resistance are classified under physiological variation. Type III resistance is the prevention of DON accumulation within spike (Miller *et al*., 1985). Type IV prevention to kernel infection and type V is the prevention of yield loss. Type IV and V are rarely

used due to the difficulty in understanding the resistance mechanisms (Mesterhazy, 1995).

Type I and Type II are the resistant types that are being used in evaluating breeding programmes and dependent on the purpose of the subjective. Type III is the third frequently used quantitative method were DON concentrations are being measured (Bai *et al*., 2001). All five of these resistant types are associated with QTLs (Anderson *et al*., 2001; Browne and Brindle, 2007; Li and Yen, 2008). QTL analysis is a reliable method to use to screen for genomic regions that can provide efficient resistance. The markers that are linked to those regions can be used in MAS without the need of environmental consideration (Buerstmayr *et al*., 2009).

2.9. Inoculation techniques

Inoculation is the deliberate introduction of a pathogen to the host. By inoculation the effect of the host-pathogen interaction can be studied to determine the resistance response. This interaction is important to assess the disease resistance in new pre-breeding lines. This concept led to the development of different inoculation techniques to test and screen pre-breeding material for FHB resistance. Spray and point inoculation is the two most frequent used methods (Mesterhazy, 1995).

2.9.1. Spray inoculation

Spray inoculation is the physical spray of a spore suspension at the anthesis stage (Miedaner *et al*., 2003). It can be used to assess type I and II resistance. It is less labour intensive compared with point inoculation when a large field experiment is evaluated. It resembles the natural host-pathogen interaction. The inoculum suspension can be sprayed more than once and the concentration can differ based on *Fusarium* spp. (Dill-Macky *et al*., 2003). *F. culmorum* and *F. graminearum* is the two species that is highly pathogenic on small-grain cereals (Edwards, 2004).

2.9.2. Point inoculation

Point inoculation is the injection of a spore suspension via a pipette to a single floret (Schroeder and Christensen 1963; Engle *et al*., 2003). By using a pipette, equal

amounts of inoculum can be injected, preventing invalid results. The volume injected vary between 5-20 µl and the spore concentration is based on pathogenicity of the *Fusarium* spp. (McCallum and Tekauz, 2002; Imathiu, 2008). Point inoculation is more labour intensive and time-consuming compared to spray inoculation. Point inoculation is mainly used to evaluate type II resistance (resistance to pathogen spread) (Miedaner *et al*., 2003). The stable nature of type II resistance makes it more favourable to evaluate FHB in wheat breeding (Burlakoti *et al*., 2010; Bai and Shaner, 1994). Point inoculation is also the method of choice for glasshouse trials (Miedaner *et al*., 2003). After the spore suspension is injected into a single floret, the injected wheat plant is placed in a dew chamber for a period of 72 h post-inoculation. Bagging the head is also an important step to create controlled humidity (Mesterhazy *et al*., 1999).

2.10. FHB assessment

Disease assessment is scoring based on a disease severity scale. One dominating rating system was the Horsfall-Barraett scale (H-B scale) developed by Horsfall and Barratt (Barratt and Horsfall, 1945). Later the scale was adapted by other research groups (Pernezney *et al*., 2003). A disease severity scale used in this study is based on the percentage of diseased spikelets over the total number of spikelets (Figure 2.10), developed by Stack and McMullen, (1995). A spikelet is defined as 3 to 5 florets on either side of the head (Engle *et al*., 2003).

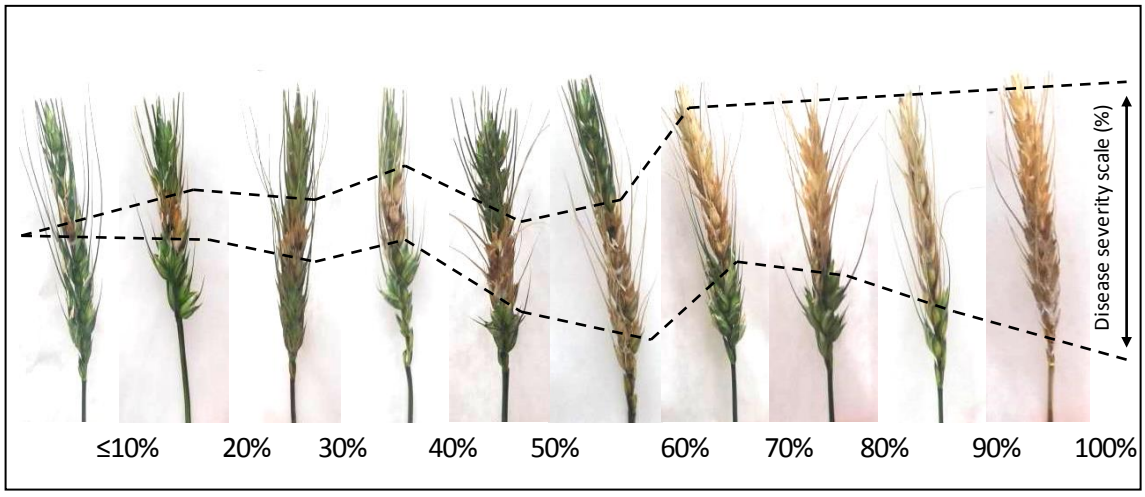


Figure 2.10. FHB severity scale was (adapted from Engle *et al*. 2003)

2.11. Breeding strategy

2.11.1. Single seed decent (SSD)

Single seed decent was developed to speed up the breeding programme and reduce the loss of genotypes during segregating populations. The method is performed by growing plants in the greenhouse under favourable and optimum conditions. A large F_1 population is being grown to insure optimum recombination of the parental chromosomes. A single seed per plant is randomly selected and used for the next generation, until optimum inbreeding is achieved. The selection process is only performed at the F5 or F6 generation (Acquaah, 2012). The advantages and disadvantages of single seed decent (SSD) is listed in table 2.6.

Table 2.6. The advantages and disadvantages of single seed decent (SSD) (Acquaah, 2012)

Advantages	Disadvantages
Homozygosity is achieved in 2-3 years	Natural selection is eliminated
A small space in greenhouse is occupied	Selection is done by phenotype and not performance
Natural selection is eliminated	Poor or no seed germination will result in F2 plant not being represented in the breeding population
Breeding programme is reduced	Selection of single seeds risk the loss of valuable genes

2.12. Male sterility systems

Male sterility is naturally found in nature. This is but a rare event and different approaches have been developed to create a male sterile line for hybrid production. It is a challenging process, but hybrids with significant amount of homogeneity of the crop of interest can be achieved to add benefits to the famers, growers and consumers (European seed association, 2013).

2.12.1. Cytoplasmic male sterility

Cytoplasmic male sterility (CMS) can either occur spontaneously following mutagenesis or it can occur through interspecific, intraspecific and intergeneric crosses (Kaul, 1988). The mitochondria determine the CMS of most angiosperm species (Hagemann, 2004). CMS is the most frequently used sterility approach; it has been used in more than 150 plant species of which wheat is one of them (Schnable and Wise, 1998).

The sterility system works with nuclear genes called fertility restorer (*Rf* or *Fr*) genes. These restorer genes are different for each CMS system. Sterility is kept by crossing the lines with a related line which is also called the maintainer line. The line of interest and the maintainer line have both identical nuclear genotypes, expect that the maintainer line contain a fertile cytoplasm. What prevent fertile in progeny is the restores genes of the maintainer lines. A recessive (*rf*) restore gene expresses male fertile lines, but sterile progeny when crossed with a sterile CMS plant (Figure 2.9) (Kempe and Gills, 2011).

Figure 2.11, explains how CMS is achieved by independent inheritance of nuclear and mitochondrial genes. The brackets indicate the genotypes of the plants. The Mother line contain a sterile cytoplasm indicated with an 'S'. To maintain this cytoplasm sterility the mother line is crossed with a maintainer line with an identical nuclear genome (Nuc: rf/rf) and a neutral (N) cytoplasm. If fertility wants be restored in the hybrid progeny the mother line is crossed with a father line carrying homozygous alleles of the nuclear genome (Nuc: Rf/Rf). The cytoplasm can either be neutral (N) or sterile (S) (Budar and Pelletier, 2001). Cytoplasmic male sterility is a complex system. CMS is only used for hybrid seed production if a CMS line and fertile restorer father line are available. CMS is very sensitive to environmental factors (Kaul, 1988).

2.12.2. Chemical hybridizing agents (*CHA*)

Chemical hybridizing agents describe the class of chemicals responsible for male sterility in breeding lines. The mode of action and the dosage play an important role in male sterility (McRae, 1985). What gives this sterility system an advantage is that male sterility can be achieved by simply spraying the female inbred parents. CHA also make it possible to produce a significant amount of parental combinations for germplasm combining ability. CHA is however only used for hybrid seed production if the chemical induces male fertility. Abiotic factors influence the efficacy of CHA significantly. Time and period of application should be carefully decided to eliminate the most abiotic influences (Cisar and Cooper, 2002).

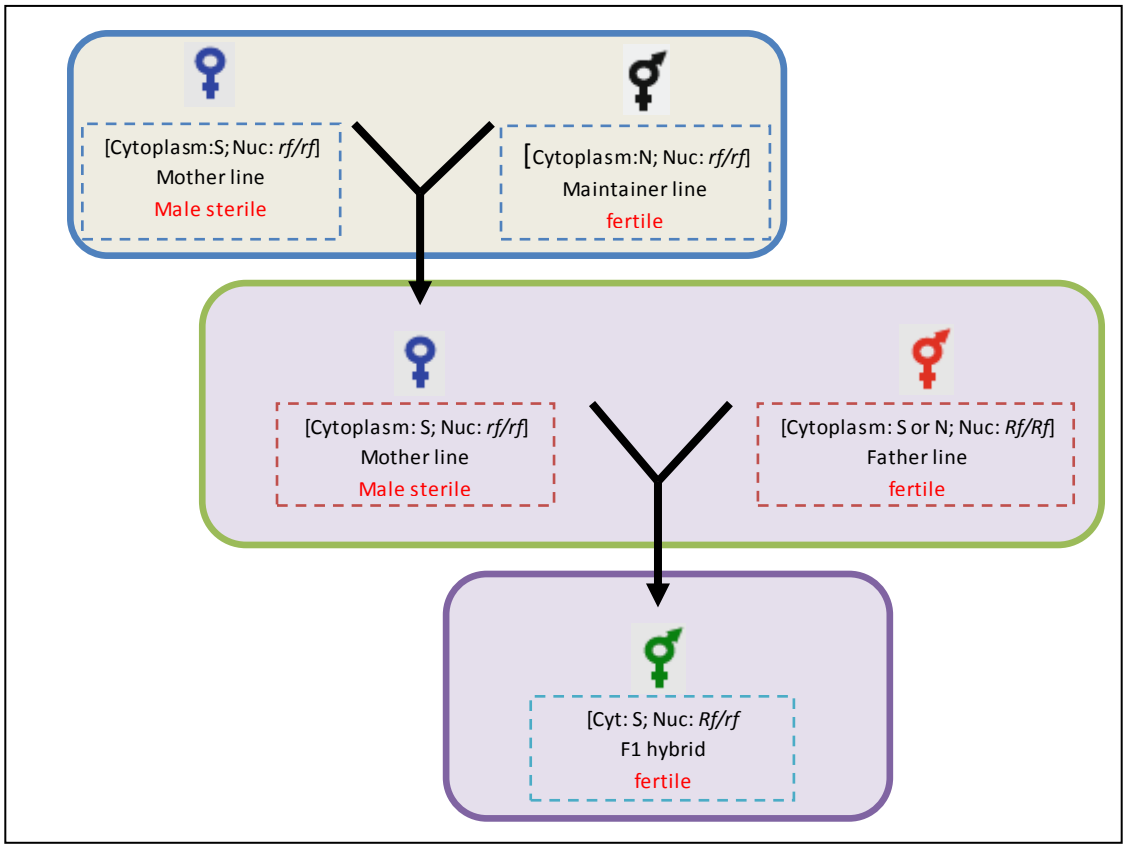


Figure 2.11. Genetic pollination control system adapted from (Budar andPelletier, 2001)

A chemical previously used in South Africa were WL84811 (azetidine-3-carboxylic acid) from Monsanto called Genesis®. Genesis® was later discontinued because of toxic residue found in F1 seed. Genesis® was used by the USA and Europe for wheat hybrid production in 2007 (Parodi and Gaju, 2009). The only CHA being used by Europe is the Croisor®100, which is a plant growth regulator (EFSA, 2010).

2.12.3. Genetic male sterility

Genetic male sterility (GMS) is caused by mutations in the nuclear-encoding genes. GMS can occur either spontaneous or due to exposure to physical or chemical mutagens. A chemical mutagen that is frequently being used is ethylmethane sulfonate (EMS). EMS induces wheat containing *ms1d*, *ms5* and *Ms3* (Fossati and Ingold, 1970; Driscoll and Barlow, 1976 and Sasakuma *et al*., 1918). The male sterility genes are either dominant or recessive and only three are known and mapped to specific chromosomes. The first gene is the *ms1*-gene which is located on chromosome 4A. The disadvantage of the *ms1*-gene is that it does not induce sufficient sterile plants that can be used in breeding techniques like recurrent selection (Whitford *et al*., 2013). The

second gene is the *Ms2*-gene. It is a dominant gene and is located on chromosome 4DS. *Ms2* was found in mutant wheat line 'Taigu' in China in 1972. *Ms2* has been widely used in breeding programmes to develop cultivars with improved adaptation and quality (Liu *et al*., 2001; Zhai and Lui, 2009).

The male sterile gene and also the gene that is going to be used in this study is the *Ms3*-gene. This male sterility gene was found after the seed of 'Chris' was treated with EMS. The gene is capable of dominant male sterility in euplasmic and alloplasmic wheat cultivars (Maan and Williams, 1984). It is found on chromosome 5AS and is closely situated to the centromere. The advantage of *Ms3* is that the expression is stable, if the growing conditions of the plants are controlled with temperatures between 16°C-25°C. The disadvantages of *Ms3* are, the selection process for cross-pollination is not adequate when grown in the field, male sterile ears are known for their low seed set, selection needs to be done before flowering and cross-hybridization is difficult. This dominant male sterility gene has been utilized in breeding programmes, including recurrent selection for population and pyramiding of useful genes (Deng and Huang, 1993).

An example of a recurrent selection population in wheat is the one that was established at the SU-PBL (Figure 2.12) (Botes, 2001). The male sterility *Ms3*-gene was used in a recurrent mass selection breeding technique to improve cross-hybridization species in combination with marker assisted technology. With the difficulty in cross-hybridization, Botes, (2001) developed a hydroponic system for large scale random inter-crossing of the selected plants. The benefit of GMS in a recurrent selection population especially for cross-hybridization species is that the amount of desirable alleles is increased while the variation in a breeding population is kept (Cao *et al*., 2009). With this in mind superior genotypes can be developed that can be used in other breeding populations lacking that desirable gene of interest (Hallauer, 1981). The overall goal was to use this breeding technique combined with the male sterility gene *Ms3* to increase genes and to maintain genetic diversity.

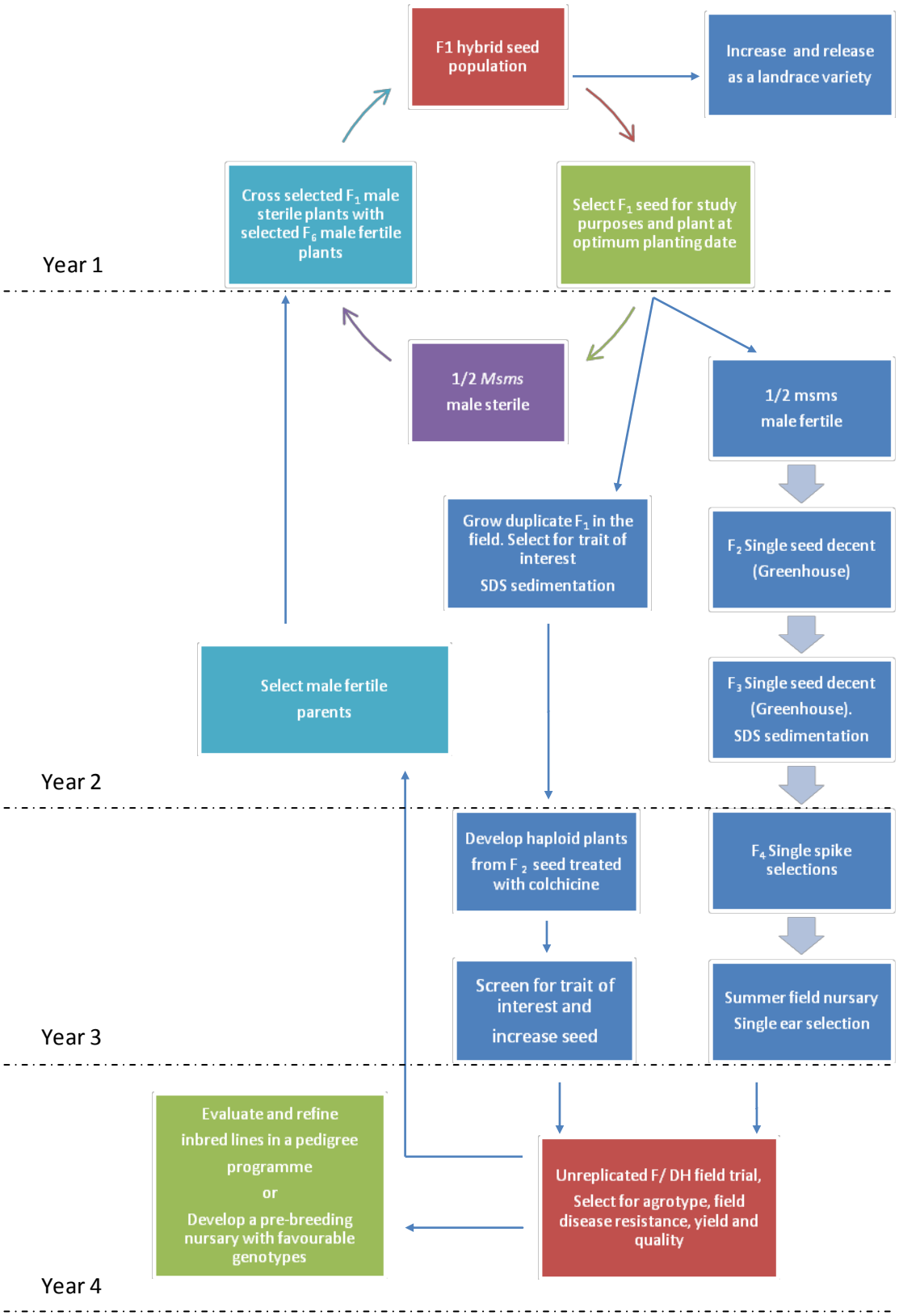


Figure 2.12. Common wheat recurrent mass selections scheme (Marais and Botes, 2009).

This breeding proposal of Botes, (2001) was very successive in raising major resistance against the devastating diseases caused by the *Puccinia* spp. stem, leaf and stripe rust. The breeding approach was used to pyramid desirable genes with durable resistance. The loss of desirable genes is limited because the breeding approach is a population improvement strategy. Selection bottlenecks are limited resulting in intense recombination and exploitation (Marais *et al*., 2000). One disadvantage is that major genes can mask the presence of genes with lesser phenotypic expression. The aid of markers however in this breeding approach of Botes, (2001) made this disadvantages not that a big of a concern anymore. Through marker assisted selection all genes can be identified in a genotype whether the genes are major or minor.

2.13. Molecular marker technology

2.13.1. Marker assisted selection

Marker assisted selection (MAS) is a modern selection technology that can be used to select a desirable traits. The trait of interest is linked to a marker. The marker and the trait (gene or QTL) of interest should be tightly associated to each other for MAS to be successful. MAS accelerate the selection process and the release of new varieties (Perez-de-Castro *et al.,* 2012). MAS are used by numerous public and private sectors on different studies. One example is where MAS was used to select against undesirable yellow flour colour (Landjeva *et al*., 2007). One of the major applications of MAS is in gene pyramiding. Gene pyramiding is extremely difficult through conventional breeding. With the aid of MAS, numerous desirable genes from different parental populations can be pyramided in to a single genotype (Ye and Smith, 2009).

Many DNA based markers have become available (Varshney *et al*., 2006). Restriction fragment length polymorphism (RFLPs). RFLPs are co-dominant and were developed to help map the human genome, but later it was adapted for plant genomic studies. One disadvantage of RFLP is its time consuming and intensive (Poehlman and Sleper, 1995). Random amplified fragment length polymorphisms (RAPDs). RAPDs are random designed primers that amplify different regions of a genome (Gupta *et al*., 1999). RAPD

technology does not need the targeted DNA information, making it a useful marker system to use on crop or biological systems that have not been tested before have been used on several crop and biological systems (Najimi *et al*., 2002).Amplified fragment length polymorphism (AFLPs). AFLP is derived from RFLP and RAPD technology providing a higher level of polymorphisms in less amount of time. AFLP is a great marker for gene tagging and mapping of plant with a large genome (Gupta *et al*., 1999).

2.13.2. Sequence characterised amplified regions (SCARs)

SCARs or sequence-tagged sites (STSs) are either dominant or co-dominant and are developed from other markers such as AFLP, RAPD or RFLP (Gupta *et al*., 1999). SCARs are characterised by low levels of polymorphism and the products are digested with restriction enzymes if no variation among products are visible (Farooq and Azam, 2002).

2.13.3. Simple sequence repeats (SSRs)

SSRs are two to six bp in length, co-dominant DNA markers that are PCR-based and are present in eukaryotic genomes (Chen *et al*., 2003). The polymorphism level of SSRs, depend on the number of repeats present on the locus throughout the genomes for animals or plants (Jonah *et al*., 2011). SSRs advantages are being efficient, stable, co-dominant, abundant, distributed evenly throughout genomes and with higher levels of polymorphism (Holton, 2001). Molecular maps of wheat are available to advance gene tagging (Röder *et al*., 1998; Stephenson *et al*., 1998). This lead to the establishment of expressed sequence tags (EST). Numerous sequencing projects have been initiated on gene discovery and data are available online (Rudd *et al*., 2003). The EST data can be used to scan online genebanks for the identification of SSRs (Varshney *et al*., 2005).However, SSRs are expensive to develop and not economic for developing complex maps (Karaoglu *et al*., 2005).

2.13.4. Single nucleotide polymorphism (SNPs)

SNPs have become extremely popular in plant breeding due to their high abundance within the genome and their high-throughput detection. SNPs are less polymorphic

due to the biallelic nature when compared with SSRs (Mannadov *et al*., 2012). The problem arises when larger more sophisticated genomes are studied (Meyers *et al*., 2001). SNPs can create supersaturated genetic maps which add value to genome-wide tracking of targeted regions.With next generation sequencing technology complex genomes can be easily screened through SNPs (Berkman *et al*., 2012). Multiple high-density SNP arrays have been successfully used to study important staple crops (Song *et al*., 2013). Genome-wide association studies (GWAS) in rice were conducted successfully by identifying valuable alleles associated with good agronomical traits (Zhao *et al*., 2011). The wheat genomes of *T. turgidum* and *T.aestivum* are still the most challenging genomes to analyse, but through the SNP iSelect array the difficulty has been reduced. The array proofed to be a promising tool for diversity studies of polyploidy wheat (Wang *et al*., 2014)

2.13.5. Diversity Arrays Technology (DArT)

DArT is based on microarray hybridization. The hybridization is build on the presence versus absence of unique DNA fragments (Wenzl *et al*., 2004). Through this array; DArT markers that are genetically distinct can be identified and arranged into a genotyping array (Gupta *et al.,* 2008).In a single array analysis several thousand loci can be typed, creating a whole-genome DNA fingerprint with several hundred data points in one assay (Jaccoud *et al.,* 2001). DArT have been successfully used in wheat (Akbari *et al*. 2006; Semagn *et al*., 2006).

2.14. Gene pyramiding

The concept of gene pyramiding is to pyramid resistance in front of *Puccinia* spp. The success however will only be significant if breeders follow the same global rust initiative (Ayliffe *et al*., 2008). Gene pyramiding involves the effectiveness of paring race-specific resistance (R) genes, adult plant resistance (APR) genes or a combination of both (Figure 2.13) for durable resistance in one genotype. Different combinations of rust resistance have been published those that do not act additively (*Sr24* and *Sr26*) and those that do (Ellis *et al*., 2014). Race-specific resistance have a tendency to be

short lived and pathotype specific. Pathogens can be unrecognisable by the host when pathogens mutate (Lagudah *et al*., 2006 and Pretorius *et al*., 2007).

Adult plant resistance is resistance in mature plants and have two advantages over R-genes. Firstly it is not pathotype specific; it gives effective resistance among all rust pathotypes. Secondly it provide durable resistance of epistatic nature which is low levels of resistance, examples are *Sr2* and *Lr34* who remained durable for over 50 years (Singh *et al*., 2005). Combining APR and R-genes in breeding programmes can improve durability. More sophisticated strategies are being used to maintain rust resistance. With the help of molecular markers these strategies are significantly enhanced (Ayliffe *et al*., 2008). Examples of successful cultivars containing combined APR and R-genes have been reported in cultivar 'Ciano 79' and 'Papago 86'. The resistance was based on *Lr16* in combination with two adult plant resistance genes with near-immune response (Sing and Huerta-Espino 1995). This is just one example which highlights the importance and significance APR has in combination with moderately race specific resistance under favourable disease incidence (Singh *et al*., 2011).

Figure 2.13. Race-specific resistance (R) genes, adult plant resistance (APR) genes or a combination of both

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

The aim of the study was to pyramid rust resistance genes and FHB resistance QTLs (leaf, stripe, stem and FHB QTL) into a single genotype using the MS-MARS scheme in combination with MAS and QTL validation. Figure 3.1 illustrate the workflow during the study and table 3.1. show the planting date and material for the MS-MARS cycles.

A segregating F_1 population was secured from SU-PBL existing pre-breeding programme. This formed the base population of this study. The base population contained rust resistance genes (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34, Lr37* and *Lr19*). The resistance was accumulated through numerous RMS cycles (Marais and Botes, 2009). The pollen donor populations were from the SU-PBL's 2014 nursery, FHB resistant material from collaborators and DH lines with rust resistance from Smit. (2013).

DNA extractions were performed on all the plants. The base populations of MS-MARS cycle 1 and MS-MARS cycle 2 were screened with the assigned markers for rust resistance genes routinely used by SU-PBL. The rust resistance genes in the male population were already validated from a previous study (Springfield, 2014). The Fusarium donor lines (Sumai cross 1, Sumai cross 2, Sumai cross 3, Sumai cross 4, Sumai cross 5 and 'Sumai 3') were screened with flanking SSR markers linked to the FHB QTLs of interest and the DH lines were validated for the presence of rust species translocations through gel electrophoresis.

Two sub-populations were performed simultaneously during MS-MARS cycle 1. The first cross was between the MS-MARS nursery and the SU-PBL 2014 male nursery. The second cross was between the male sterile plants from the MS-MARS nursery and the fusarium donor lines. The F1 seed obtained from both cross-pollinations were harvested and planted again to form the second MS-MARS segregating population. The pollen donors for the second cross were a combination of FHB, double haploids and a SU-PBL 2015 nursery. Male fertile ears from the second MS-MARS segregating population were harvested to be used for single seed decent (SSD) and QTL validation. Genomic DNA was extracted from the F2-SSD material and screened for the presence of FHB QTL. The selected plants were cloned, one for SSD and one for QTL validation.

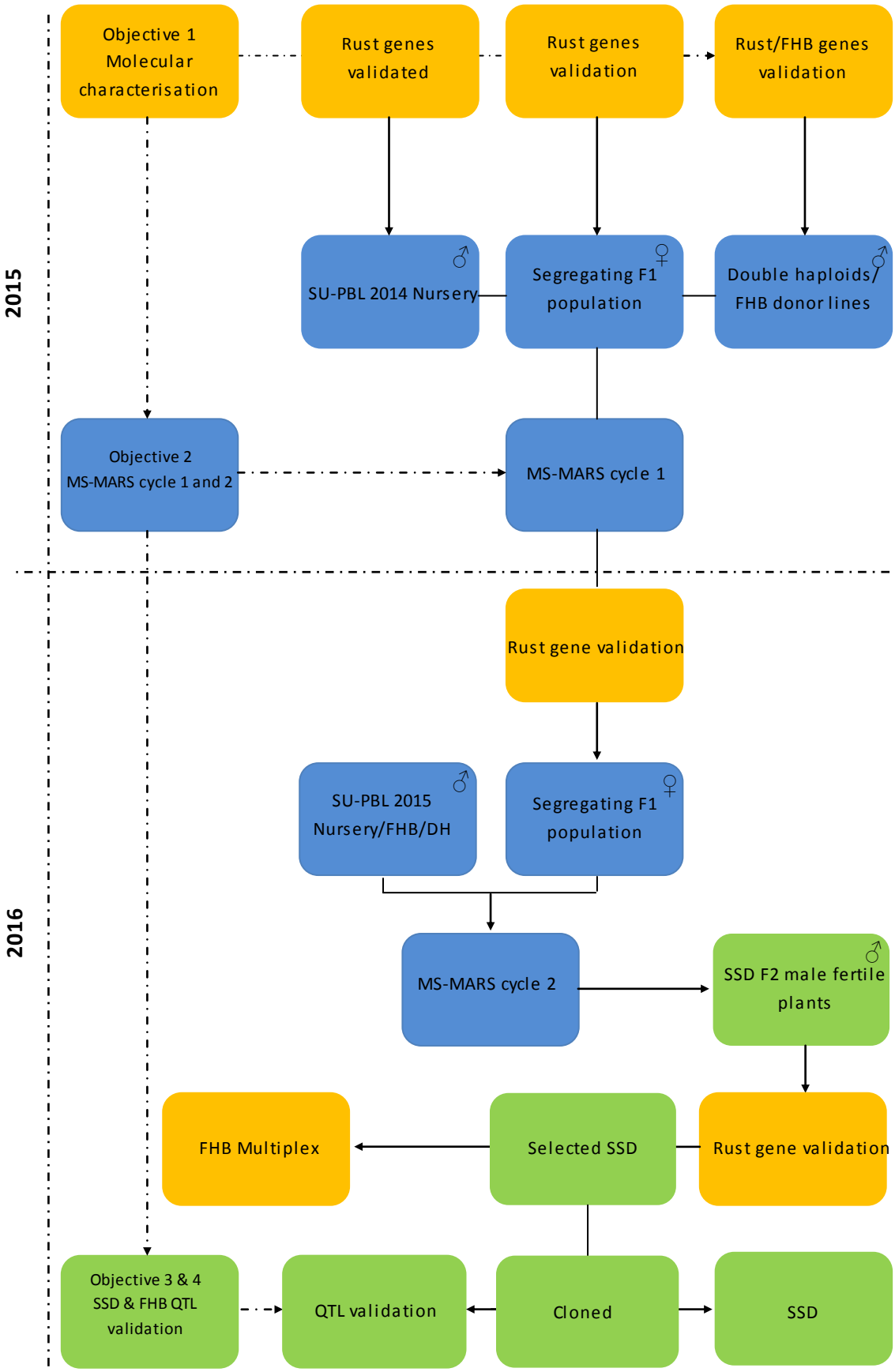


Figure 3.1 An illustration of the workflow during the study

Table 3.1. Planting date and planting material for the MS-MARS cycle 1 and 2

MS-MARS cycle 1	Planting date 2015	Planting material
Male sterile	9th,14th,16thand 21st of April	SU-PBL 2014 Nursery
Male fertile	9th,16thand 23rd of April	SU-PBL 2014 Nursery
FHB fertile	10th,17th and 24th of April	FHB lines from collaborators
MS-MARS cycle 2	Planting date 2015	Planting material
Male sterile	27th of Oct, 5th of Nov, 9th of Nov, 13th of Nov, 17th of Nov	SU-PBL 2015 Nursery
Male fertile	5th, 9th, 13th and 17th of Nov	Double haploids, FHB and SU-PBL 2015 Makatini

*No pedigree is given due to the confidentiality of SU-PBL and the University.

The male fertile lines were harvested after the cross-pollination of the second MS-MARS cycle. The 853 seed obtained were used for SSD and was planted on the 30th of March 2016. The SSD population was screened for the presence of FHB QTL and rust resistance genes. Only the SSD lines with FHB QTL and rust resistance genes were selected for cloning. The selected plants were cloned on the 4th of June 2016 one for QTL validation via point inoculation and one plant for SSD. The clones for QTL validation were replanted in pots and placed in a randomized complete block design. The two control lines that were used in this study were included during the QTL validation trial one resistant line ('Sumai 3') and one susceptible line ('SST027'). The clones for SSD were replanted back in there original pots. Based on the best performing lines after QTL validation the corresponding SSD was selected and used in the recurrent pre-breeding programme at the SU-PBL.

3.2. DNA extraction

3.2.1. Genomic extraction of leaf material

Plant material were cut into small 2 mm pieces and placed into 2 ml micro centrifuge tubes. The following extraction chemicals were added [500 µl of 2 % (CTAB) cetyltrimethylammoniumbromide buffer [100 mM Tris-Cl (ph 8.0) 1.4 M NaCl, 20 mM EDTA (pH 8.0)]. The plant material together with the CTAB buffer was homogenized by a high-speed TissueLyser II. Three bearing balls was added in each tube and shaken 3 times at 30 Hz for 30 sec. The samples were held in placed by the two clamps at the

front of the TissueLyer. The total gDNA of the leaf material was isolated using a short DNA extraction protocol by Doyle and Doyle (1990) (Figure 3.2).

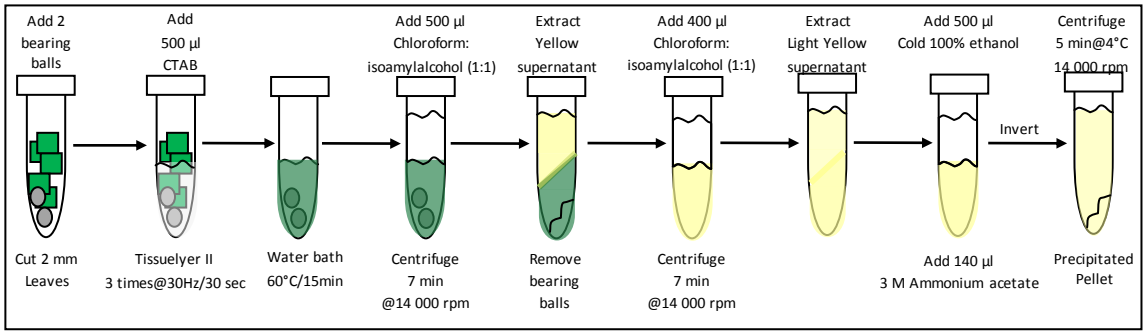


Figure 3.2. DNA extraction protocol by Doyle and Doyle (1990)

The ethanol (100 %) was cast out leaving only the precipitated pellets behind in the tubes. The pellets were washed with 70 % ethanol by centrifuge @ 12 000 rpm and cast out for the pellets to air dry (+/- 3 hours). The air dried pellets was suspended in 30 µl distilled water (dH_2O). The suspended DNA was stored at -20 °C. The DNA concentration was measured with the Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Kempton Park, RSA). Thirty microliter dilutions with a DNA concentration of 100 ng/µl were made up in 0.5 ml tubes from all of the DNA samples. The $C_1V_1=C_2V_2$ was used to calculate the final DNA concentration

3.3. Molecular screening of wheat lines

All PCR reactions were performed in multiplex except for *Sr2*, *Lr34* and the FHB markers. A 2720 Thermal Cycler (Applied Biosystems, Fairland, RSA) was used during all of the PCR reactions that were performed. Bioline (distributed by Celtic Molecular Diagnostics (Edms) Bpk, Mowbray, RSA) supplied the magnesium chloride and KapaBiosystems (distributed by Lasec SA (Pty) Ltd, Cape Town, RSA) the KAPAGreen Readymix and KAPA2G^{TM} fast PCR Mix.

3.3.1. Screening crossing parents for rust resistance genes in the MS-MARS scheme

The microsatellite markers used to screen the genes of interest specific for this study were bought from Integrated DNA Technologies (Whitehead Scientific Inc, Stikland,

RSA) which are listed in table 3.2. Molecular markers for *Sr2*, *Sr31, Lr34*/*Yr18*/*Pm38*, *Lr24*/*Sr24*, *Lr37*/*Sr38*/*Yr17*, *Sr26* and *Lr19* were used to screen the females (1:1 segregating MS-MARS base population prior to cross-pollination). The microsatellite markers formed part of the standard panel of markers used by the SU-PBL for the detection of particular genes in the MS-MARS population (Wessels and Botes, 2014).

The Multiplex PCR reaction contained a final volume of 20.7 µl: 12.5 µl one time KAPA2G™fast PCR Mix, 0.5 µl of the 10 µM forward primer and 0.5 µl of the 10 µM reverse primer. One exception was for the 12C primer with 1 µl of the 10 µM forward primer and 1 µl of the 10 µM reverse primer. To make up the final volume add 1.2 µl gDNA. All PCR products were separated on a 1.8 % Agarose gels at 100 V in 1 X TBE buffer [200 ml: 5 X TBE stock solution (0.5 M Tris (hydroxymetyl) Aminomethane, 0.5 m Boric acid, 0.5 M Ethylenediamine tetra acetic acid disodium salt dehydrate (EDTA) and 800 ml of dH_2O to bring the final volume to 1 L].

The PCR reaction for*Sr2* contained a final volume of 12.9 µl: 7.5 µl one time KAPAGreen ready Mix, 0.45 µl of the 10 µM *csSr2* forward primer and 0.45 µl of the 10 µM *csSr2* reverse primer, 1.5 µl gDNA and 3 µl dH_2O to bring the volume to 12.9 µl. Half 6.45 µl of the PCR products for primer *csSr2* were separated on 1 % Agarose gels at 120 V. For the enzyme cutting step, 2.5 µl of *Bsp*HI restriction enzyme were added to the other 6.45 µl of the PCR products for primer *csSr2* and incubated for 1h at 36 °C. After incubation the products were separated on 2 % Agarose gels at 120 V in 1 X TBE buffer.

The PCR reaction for *Lr34* contained a final volume of 17.6 µl: 10 µl one time KAPAGreen ready mix, 0.6 µl of the 10 µM *Lr34DINT9* forward primer, 0.6 µl of the 10 µM *Lr34PLUS* reverse primer, 0.25 µl of the 10 µM Cslv forward primer, 0.25 µl of the 10 µM Cslv reverse primer, 1.3 µl of gDNA (100 ng/µl) and 4.6 µl dH_2O to bring the volume to 16.3 µl. All PCR products were separated on 1.5 % Agarose gels at 120 V in 1 X TBE buffer.

Table 3.2. The SU-PBL standard panel of markers

Genes	Primer	Primer sequences	Ta (°C)	Fragment size (bp)	References
Lr34	L34DINT9 L34PLUS	F: 5'- TTGATGAAACCAGTTTTTTTTCTA- 3' R: 5'- GCCATTTAACATAATCATGATGGA- 3'	57	517	Krattinger *et al.* (2009)
Sr2	*csSr2*	F: 5'- CAAGGGTTGCTAGGATTGGAAAAC- 3' R: 5'- AGATAACTCTTATGATCTTACATTTTTCTG- 3'	60	53,112,172	Mago *et al*.(2011)
*Sr24**	SCS719	F: 5'- TCGTCCAGATCAGAATGTG- 3' R: 5'- CTCGTCGATTAGCAGTGAG- 3'	57	719	Cherukuri *et al.*(2003)
*Sr26**	*Sr26*#43	F: 5'- AATCGTCCACATTGGCTTCT- 3' R: 5'- CGCAACAAAATCATGCACTA-3'	57	207	Mago *et al*.(2005)
*Lr19**	12C	F: 5'-CATCCTTGGGGACCTC-3' R: 5'-CCAGCTCGCATACATCCA-3'	57	119	Prins *et al*. (2001)
*Lr37**	Ventriup Ln2	F: 5'-AGGGGCTACTGACCAAGGCT-3' R: 5'-TGCAGCTACAGCAGTATGTACACAAAA-3'	57	259	Helguera *et al*. (2003)
*Sr31**	Iag95	F: 5'-CTC TGT GGA TAG TTA CTT GAT CGA-3' R: 5'-CCT AGA ACA TGC ATG GCT GTT ACA-3'	57	1030	Mago *et al*. (2005)

* Genes analysed in one multiplex reaction

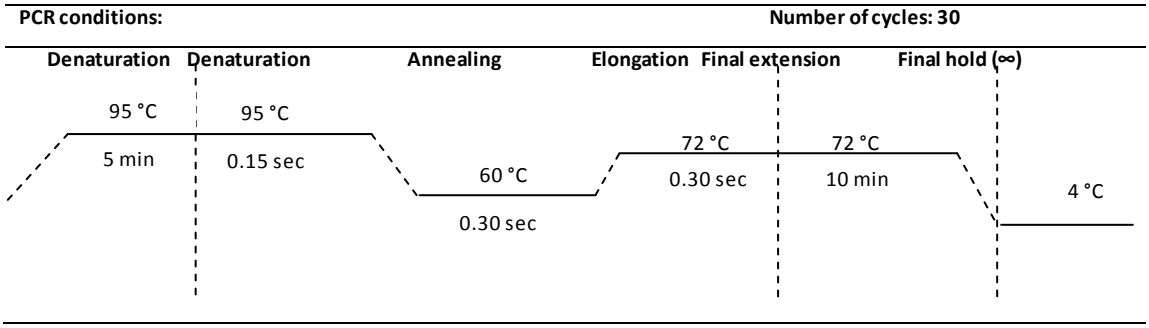
3.3.2. Validation of species resistance genes in the double haploid population

The double haploid population was validated for the presence of rust resistance translocation. The markers used to quantify the resistance translocations (*Lr53*/*Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38*, *Lr59*, *Lr62*/*Yr42*) are listed in table 3.3. The PCR reaction for the multiplex contained a final volume of 20 µl: 10 µl one time KAPA2G™fast PCR Mix, 0.5 µl of each 10 µM primer, 1 µl gDNA (100 ng/µl) and 5 µl dH_2O to bring the final volume to 20 µl. Table 3.4 list the PCR conditions for the species multiplex reaction.

Table 3.3. The markers used to quantify the resistance translocations

Translocations	Primer	Primer sequencing	Ta (°C)	Fragment size (bp)	References
Lr53/Yr35	S8N1-OF	5'-CACGTTGGTAACTGAACATT-3'	60	500	Eksteen (2009)
	S8N1-OR	5'-CTCACGTTGGACTTAAA-3'			
Lr54/Yr37	S14 275F	5'-CATGCAGAAAACGACACACC-3'	60	297	Heyns (2010)
	S14 252R	5'-GGTAAGTGGTCAGGCGTTGT-3'			
Lr56/Yr38	S8N1-OF	5'-CACGTTGGTAACTGAACATT-3'	60	500	Eksteen (2009)
	S8N1-OR	5'-CTCACGTTGGACTTAAA-3'			
Lr59	S15 T3F	5'-GTCACTTGCTTGAATTTAATG-3'	60	622	Eksteen (2009)
	S15 T3R	5'-TCCATAGCTGGTAGCTAGATG-3'			
Lr62/Yr42	Opw 7.2F	5'-CAGGAGCATAGTCATACTTGGG-3'	60	700	Eksteen (2009)
	Opw 7.2R	5'-CTGGACGTCAACAATGGC-3'			

Table 3.4. The PCR conditions for the species multiplex reaction



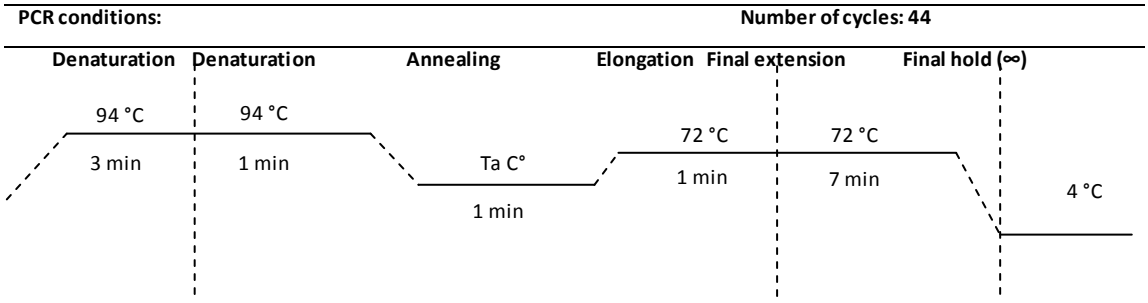
3.3.3. Screening crossing parents for Fusarium resistance QTL in the male donor lines

Markers for the FHB QTL (*Qfhs.ifa-5A*, *Fhb2-6B*, *7AQTL* and *Qfhs.ndsu-3BS*) are listed in table 3.5. The markers were used to screen the fusarium male donor lines. Each of the primer sets was performed in a single PCR reaction to validate the presence of the different microsatellite markers flanking the QTLs of interest. From the 10 microsatellite markers only 7 were selected to continue in this project. The exclusion is based on the study done by Peng *et al*. (2000), which stated that the accuracy and competency of using only one flanking marker can lead to a false positive for the presence of the QTLs. Based on this *Gwm156* were excluded, *Gwm644* and *Gwm133* which flank *Fhb2* gave unreliable results, were there for excluded in this study. The single PCR reactions contained a final volume of 14 µl: 6.25 µl one times KAPA2G™fast PCR Mix, 0.5 µl of each 10 µM forward primers, 0.5 µl of each 10 µM reverse primer, 1 µl of gDNA (100 ng/µl) and 5.75 µl dH_2O to bring to a final volume of 14 µl. Table 3.6 list the PCR cycling condition for FHB QTLs reaction. The PCR products were kept at 4°C. All PCR products were separated on 6 % polyacrylamide gels, except for *Gwm533* and *Gwm493* were separated on 1.5 % Agarose gels.

Table 3.5. The SSR markers used in this study together with the labelled dyes, fragment size, QTL and annealing (Ta) temperature (Roder *et al*., 1998)

QTL	SSR markers	Selected markers	Labeled dye	Primer sequencing	Ta (°C)	Fragment Size (bp)
Qfhs.ifa-5A	*Xgwm304*	Yes	FAM	F: 5'-AGGAAACAGAAATATCGCGG-3' R: 5'-AGGACTGTGGGGAATGAATG-3'	57	208
	Xgwm293	Yes	NED	F: 5'-TACTGGTTCACATTGGTGCG-3' R: 5'-TCGCCATCACTCGTTCAAG-3'	57	305
	Xgwm156	No	PET	F: 5'-CCAACCGTGCTATTAGTCATTC-3' R: 5'-CAATGCAGGCCCTCCTAAC-3'	57	205
Fhb2-6B	*Xgwm644*	No	PET	F: 5'-GTGGGTCAAGGCCAAGG- 3' R: 5'-AGGAGTAGCGTGAGGGGC- 3'	57	206
	Xgwm133	No	VIC	F: 5'-ATCTAAACAAGACGGCGGTG-3' R: 5'-ATCTGTGACAACCGGTGAGA-3'	57	135
7AQTL	*Xgwm130*	Yes	PET	F: 5'-AGCTCTGCTTCACGAGGAAG-3' R: 5'-CTCCTCTTTATATCGCGTCCC-3'	57	121
	Xgwm233	Yes	NED	F:5'-TCAAAACATAAATGTTCATTGGA-3' R: 5'-TCAACCGTGTGTAATTTTGTCC-3'	57	248
Qfhs.ndsu-3BS	*Xgwm493*	Yes	VIC	F: 5'-TTCCCATAACTAAAACCGCG-3' R: 5'-GGAACATCATTTCTGGACTTTG-3'	57	211
	Xgwm533	Yes	FAM	F: 5'-AAGGCGAATCAAACGGAATA-3' R: 5'-GTTGCTTTAGGGGAAAAGCC-3'	57	159
	Barc133	Yes	NED	F: 5'-AGCGCTCGAAAAGTCAG-3' R: 5'-GGCAGGTCCAACTCCAG-3'	57	125

Table 3.6. PCR cycling condition for FHB QTLs reaction



3.3.3.1. Multiplex optimization with fluorescently labelled primers

The seven selected primers were ordered from life technologies, South Africa. Only the forward (5'-3') primers were labelled with 4 different fluorescently labelled dyes blue (FAM), Green (Vic), Red (Pet) and yellow (Ned) respectively, table 3.5 lists the labelled dyes for each forward primer. The dyes were selected based on fragment size and labelled with different fluorescent dyes to prevent primers with similar fragment sizes from overlapping. The five primers were first tested on 6 % polyacrylamide gels before samples were taken to the central analytical facility (CAF) for fragment analysis.

Markers *Gwm533* and *Gwm493* were included in the multiplex send to CAF, but were not tested on the 6 % polyacrylamide gels, but on 1.5 % agarose gels.

Four multiplex PCR reactions were tested on 6 % Polyacrylamide gels. The PCR reaction with the expected fragment sizes, were the one that was selected to continue in this study. Table 3.7 list the final PCR reaction conditions during the optimization phase of this multiplex and table 3.8 lists the PCR multiplex setup of each primer.

Table 3.7. The final PCR reaction conditions during the optimization phase of this multiplex

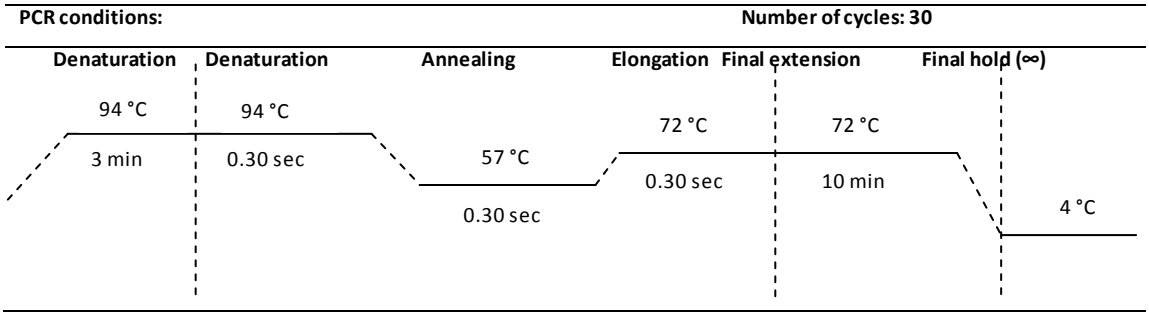


Table 3.8. The PCR multiplex setup of each primer

PCR multiplex setup	25 rxn[1]	Primers	Primer concentration
2X Kapa2G Fast Multiplex Mix	12.5		
10 µM Forward primer	1 µl	*Gwm304*-F	0.4 µM each
10 µM Reverse primer	1 µl	*Gwm304*-R	0.4 µM each
10 µM Forward primer	1 µl	*Gwm493*-F	0.4 µM each
10 µM Reverse primer	1 µl	*Gwm493*-R	0.4 µM each
10 µM Forward primer	0.75 µl	*Gwm293*-F	0.3 µM each
10 µM Reverse primer	0.75 µl	*Gwm293*-R	0.3 µM each
10 µM Forward primer	0.75 µl	*Gwm130*-F	0.3 µM each
10 µM Reverse primer	0.75 µl	*Gwm130*-R	0.3 µM each
10 µM Forward primer	0.75 µl	*Gwm233*-F	0.3 µM each
10 µM Reverse primer	0.75 µl	*Gwm233*-R	0.3 µM each
10 µM Forward primer	0.65 µl	*Gwm533*-F	0.26 µM each
10 µM Reverse primer	0.65 µl	*Gwm533*-R	0.26 µM each
10 µM Forward primer	0.65 µl	*Barc133*-F	0.26 µM each
10 µM Reverse primer	0.65 µl	*Barc133*-R	0.26 µM each
Template DNA	1.4		
Final reaction volume	25 µl		

3.3.3.2. Capillary electrophoresis

Capillary Electrophoresis (CE) is much more accurate and consistent when compared to PAGE and agarose gel electrophoresis (Koumi *et al*., 2004). CE show significant reproducibility with a deviation of only < 0.5 bp compared to a deviation of 1 bp for PAGE and > 3 bp for agarose gels (Vemireddy *et al*., 2007).The PCR products were taken to the Central analytical facility (CAF) at Stellenbosch University (www.sun.ac.za/saf) for CE.All the PCR samples were due for a standard post PCR clean-up prior to CE. Two µl of cleaned PCR product was mixed with the internal size standard (Lize250) and Hi-Di (formemide) prior to denaturation for 5 min at 95°C. The samples were placed directly after the denaturing step on ice for 5 min. The CE was either performed on an AI3130xl or ABI3730xl using POP7 (sieving medium) and a capillary array (50 cm). Applied Biosystems supplied all of the necessary reagents.

A data file were created after CE and imported to GeneMapper V5 (Life Technologies, RSA) for analysis. Panels and bins were created for possible alleles for each marker in accordance to the resistant ('Sumai 3') and susceptible ('SST027') control lines. The alleles from the control lines were used to evaluate the rest of the multiplexed samples. After evaluation the software summarises a data sheet for each sample. The data sheet was converted to a Microsoft Excel document for further analysis of the data.

3.4. Polyacrylamide gel- electrophoresis (PAGE)

3.4.1. Gel preparation

Polyacrylamide gel-electrophoresis was performed for all the microsatellite FHB markers. Three components are involved for the gel preparation. First, a 40 % acrylamide stock solution was prepared [5.3 M acrylamide, 0.129 M bis-acrylamide and 200 ml dH_2O. The bottle were covered with foil and stored at 4 °C. Secondly, a 6 % sequencing gel mix was prepared [37.5 ml 40 % acrylamide stock solution, 6 M urea, 50 ml of 5 X TBE and brought to volume with 250 ml distilled water (dH_2O)]. Thirdly, a 10 % solution of ammonium persulfate (APS) was prepared [0.1 g of APS in 1 ml of H_2O]. The final gel solution was prepared by adding [800 µl APS solution, 160 µl N, N, N‟, N‟‟-

Tetramethyl ethylene diamine (TMED) to (160 ml of the 6 % sequencing gel mix)]. The solution were thoroughly mixed and cast immediately.

3.4.2. Plate preparation

The plate preparation had two steps. Step one consisted of three dilutions. The first dilution; 125 µl of the plate glue stock solution was diluted in 25 ml 100 % ethanol. The second dilution 500 µl of the first dilution were further diluted in 1500 µl 100 % ethanol and the third dilution 1740 µl of the second dilution and 140 µl 10 % acetic acid was added to a 2 ml micro centrifuge tube. Step two; the long glass plate and short glass plates were thoroughly cleaned with 100 % ethanol, after that Wynn's C-thru was used to gently wipe the long glass plate (it prevent the plate from sticking to the gel), left for 3 minutes and wiped until thoroughly clean. The short plate was wiped with the third dilution in step one, left for 30 seconds and wiped until thoroughly clean. The spacers were placed on the long sides of the long glass plate. The short glass plate was placed on top of the long plate. The two glass plates were strapped with a casting boot to hold the plates together. After the casting boot was secured, the gel was cast and the comb where put in place and clamped tightly and left for one hour for the gel to set.

3.4.3. Loading of samples

After the PCR step the samples were loaded together with 10 µl formamide loading dye [98 % (v/v) de-ionized formamide, 10 mM EDTA, pH 8.0, 0.05 % (w/v) bromophenol blue and 0.05 % (w/v) xylene cyanol FF]. The samples were denatured with a 2720 Thermal Cycler for five minutes at 95 °C. Samples were placed on ice immediately after denaturation prior to loading. The single stranded PCR products were performed on a 6 % polyacrylamide gel.

3.4.4. Silver staining

The silver staining was done in three steps. The first step was the fixing solution: (210 ml 100 % ethanol was added to 1879.50 ml dH_2O). Just before use 10.5 ml acetic acid was added to the fixing solution. The second step was the staining solution: [2.1 g of silver nitrate ($AgNO_3$) was added to 2100 ml dH_2O]. The third step was the developing

solution: [31.5 g of sodium hydroxide (NaOH) was added to 2100 ml dH_2O]. Just before using the developing solution add 8.505 ml formaldehyde.

After electrophoresis the gel was taken off and placed on ice immediately to help with the separation of the two glass plates. After 2 min the spacers were removed and the long glass plate was carefully lifted up with a wonder wedge. The short glass plate was placed into the fixing solution and shaken for 20 minutes on the shaker. The gel was then transferred to distilled water (dH_2O) and rinsed for 5 minutes two times. After rinsing; the gel was transferred to the staining solution and shaken for 20 minutes on the shaker. The gel was then again transferred to distilled water (dH_2O) and rinsed for 10 sec. The gel was then transferred to the developing solution and shaken for 20 to 30 minutes or until bands became clearly distinctive. The developing solution was then discarded and the gel was then rinsed for 10 sec one last time.

3.5. MS-MARS

The selected male sterile and male fertile ears were cut at flowering for cross-pollination and placed in a regulated nutrient solution to gain maximum pollination. Florets on the male sterile ears were cut open to allow male fertile pollen to cross-pollination. The fertilized male sterile ears were kept in the regulated nutrient solution until the seed ripened. The nutrient solution was changed once every two weeks. The male fertile ears were removed after pollination. The trays for the regulated nutrient solution consisted of iron trays which were galvanized (Dimensions: 600 mm x 450 mm x 160 mm). The inside of the trays were painted with antifungal paint. The trays had galvanized lids each lid had the capacity to accommodate 230 male sterile ears. For the male fertile ears two slender trays that can hold oasis were designed to accommodate up to 70 male fertile ears. The trays were placed on a raised frame (600 mm high) on either side of the main tray. The main tray was designed with an inlet (steel nozzle in the front) and outlet (tap at the rear) of the tray for changing the regulated nutrient solution conveniently without removing the male sterile ears Figure 3.3. Sterile and fertile ears were cut twice a week to fill 1-2 trays over a period of six to eight weeks. Each tray was filled with 40 % nutrient solution, 0.01 % “Jik” in water (H_2O). The nutrient solution was made up of 150 g of Microplex GA, 12.5 kg of Sol-U-

Fert T3T and 100 L of water and 2: 6.8 kg of Calcinit mixed in 100 L of water. A final dilution is made by taking 14 L from the 100 L of nutrient solution and added to a 1500 L tank with water. The main trays were kept in a growth chamber with LED lighting until optimum seed set were achieved. For the prevention of fungal growth an air pump was used to aerate the regulated nutrient solution. Figure 3.3 Show the different steps during the recurrent scheme.

3.6. QTL validation

The selected plants were phenotypically screened using the point inoculation technique to confirm the presence of the SSR marker data flanking the QTLs of interest. Four FHB isolates listed in table 3.9 (W-2-574, W-2-962, W-2-952 and W-2-922) were used for the inoculum (provided by Gert van Coller). DAFFsub-cultured the Fusarium colonies on fresh PDA plates using a single spore technique from Leslie and Summerell. (2006) each isolate was stored at 23 °C. Before inoculation a spore suspension of 300 µl was made for each isolate. The spore concentration was calculated with a hemocytometer under a light microscope at 40x magnification. Figure 3.4 represent the grid on the hemocytometer which was designed to count the number of spores in a 1 mm^3 area. The 1 mm^2 area is further divided into 0.25 mm x 0.25 mm squares, giving a total of 25 squares/1 mm^2. The spores were counted in the five blue squares, multiplied by 25 and multiplied by 0.1 (1 mm^3 = 0.1 µl) for the concentration of spores/0.1 µl. The spore concentration calculated for 0.1µl was used to determine the spore concentration of the 300 µl suspension. The suspensions were then diluted to a final concentration of 5 x 10^4 spores.ml^{-1}. The $C_1V_1=C_2V_2$ formula was used to do the calculations. The four isolates were added together with a final volume of 1ml and used as inoculum. The rest of the suspension was stored at 4°C for future use.

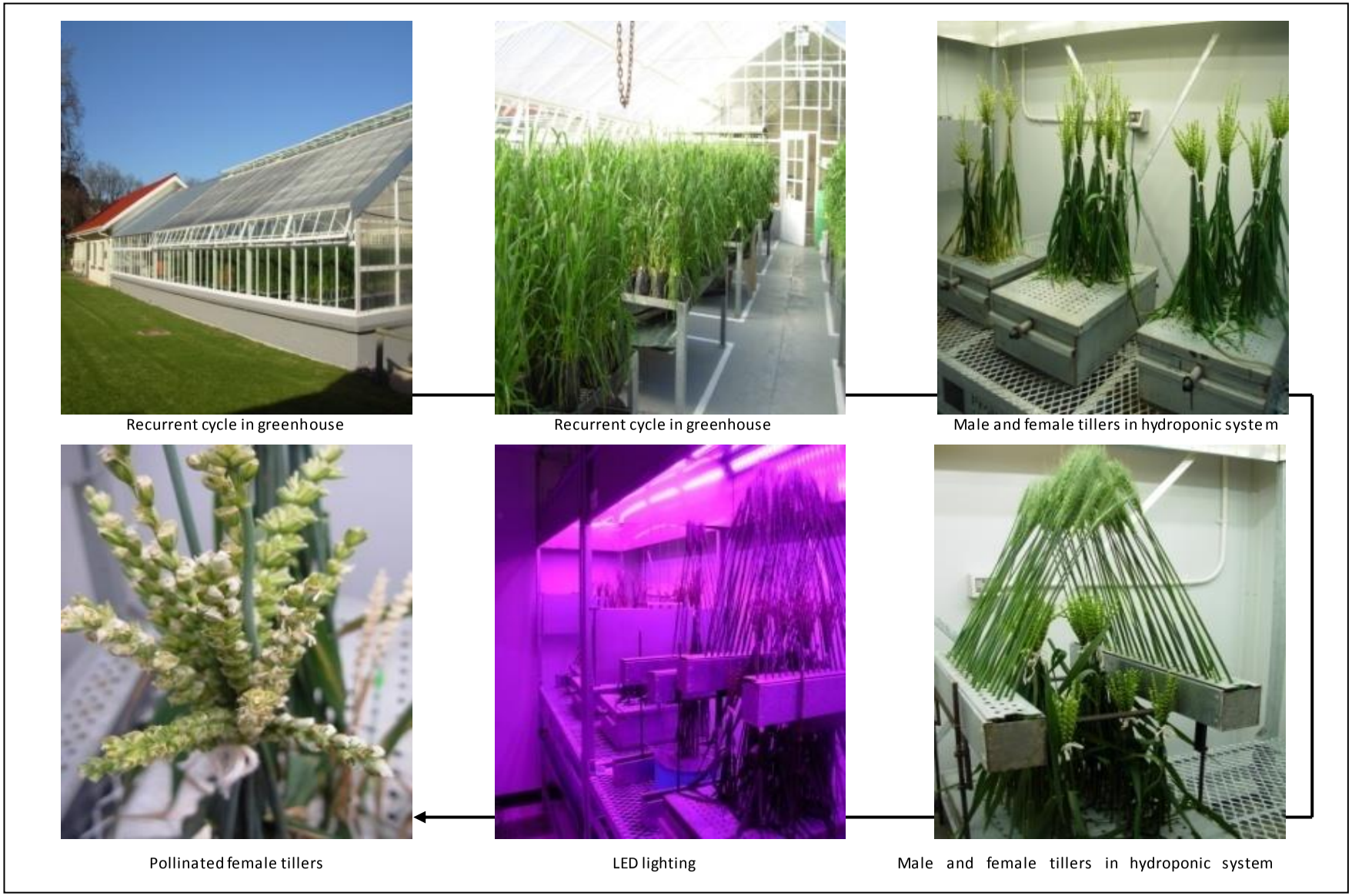


Figure 3.3. Show the different steps during the recurrent scheme

Table 3.9. The four Fusarium isolates used in this study

ID number	Host	Cultivar	Province	Locality	Year	Species	Chemotype
W-2-574	Wheat	Kariega	Mpumalanga	Groblersdal	2009	*F. graminearum*	15-ADON
W-2-962	Wheat	Kariega	Northern cape	Barkley-Wes	2009	*F. graminearum*	15-ADON
W-2-952	Wheat	Baviaans	Northern Cape	Hopetown	2009	*F. graminearum*	15-ADON
W-2-922	Wheat	Kariega	Northern Cape	Bull Hill	2009	*F. graminearum*	15-ADON

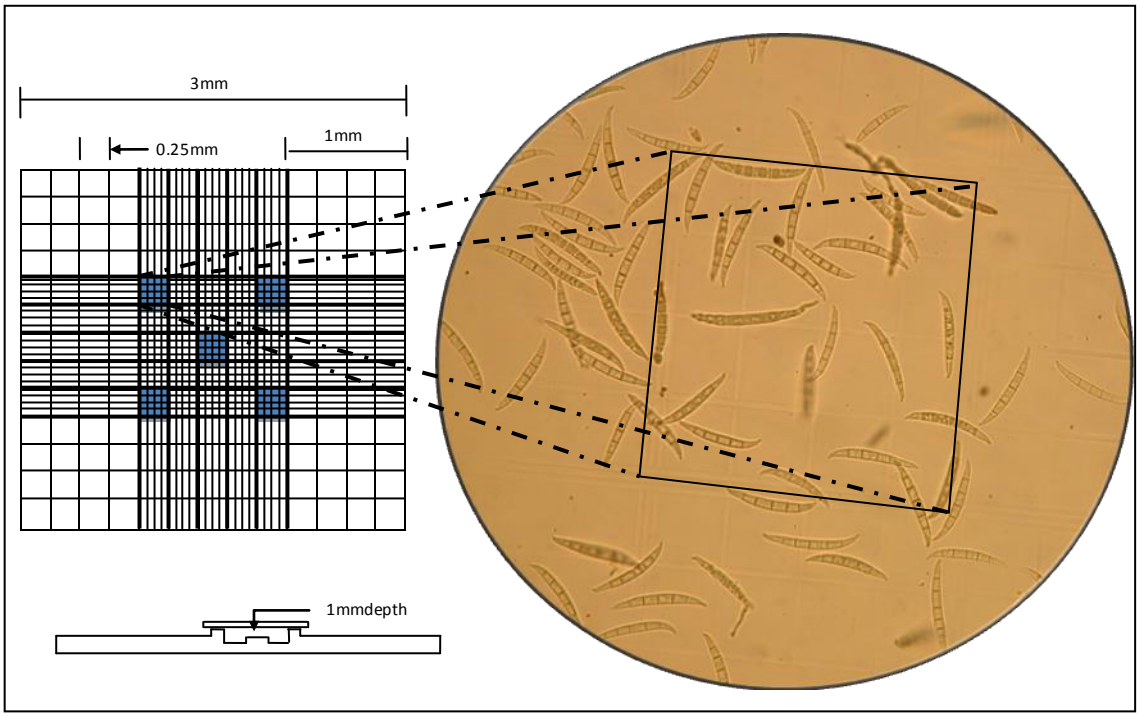


Figure 3.4. Calculating the spore concentration under a microscope with a hemocytometer

3.6.1. Point inoculation

Grausgruber *et al*. (1995) reported the wheat plant to be susceptible at anthesis. There for each of the selected plants was inoculated at anthesis via the point inoculation method, but instead of using a pipette or syringe a 2 mm x 2 mm cotton wool, dipped in the spore suspension was used. The cotton wool was chosen to prevent the spore suspension from running out of the inoculated floret. Each inoculated ear was covered with a bag. The plants were then placed in a dew chamber at 22-24°C with 100 % relative humidity for 24 h. After 24 h the plants were taken out and put back in the greenhouse. Figure 3.5 Represent the steps from inoculating the florets to evaluating the inoculated florets.

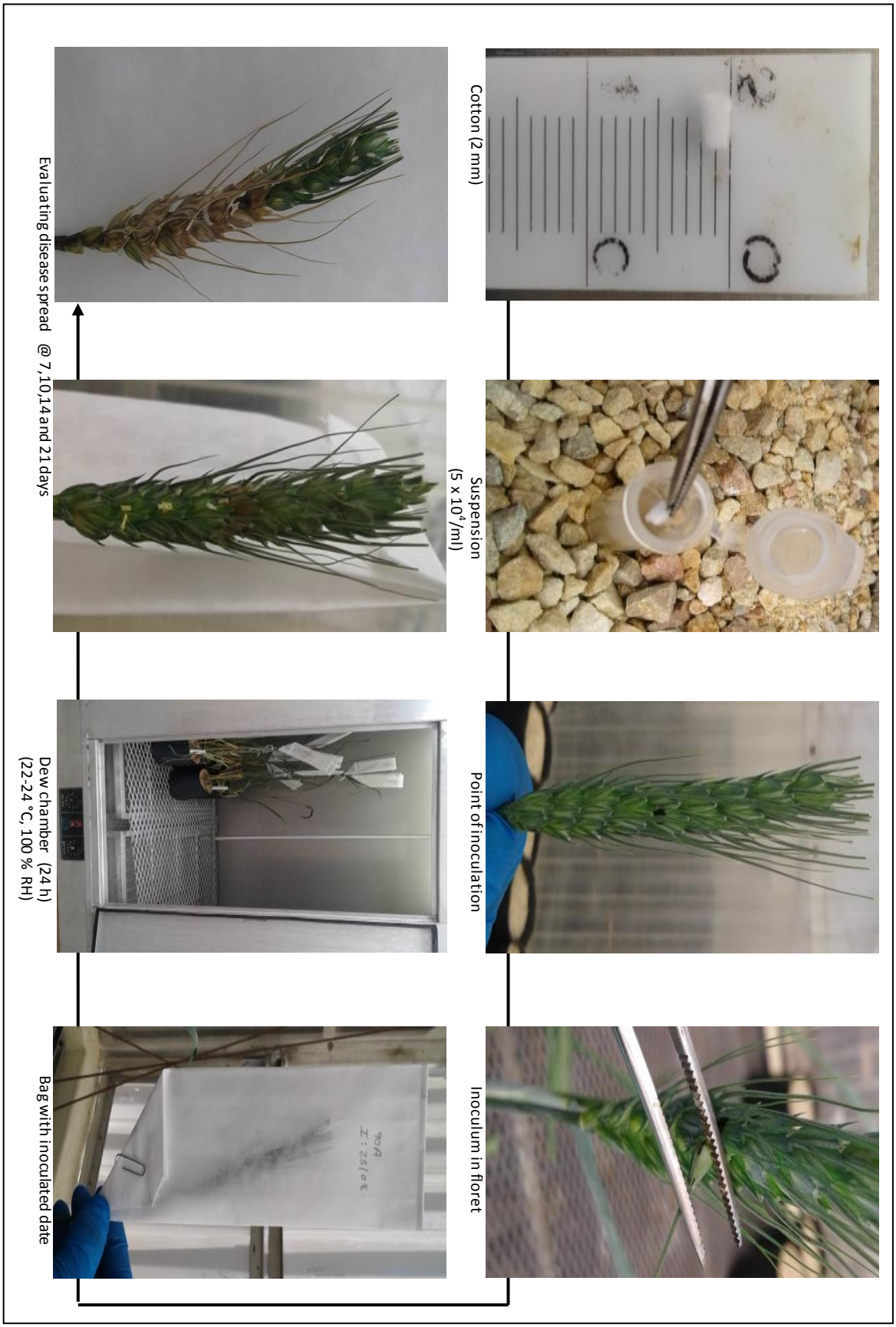


Figure 3.5. Represent the steps from inoculation to evaluating the inoculated ears

3.6.2. Rating the disease

The disease severity was rated by the following equation (Total number of infected florets ÷ Total number of florets X 100 = %) The disease severity was evaluated post-inoculation on the 7^{th}, 10^{th}, 14^{th} and 21^{ste} dpi for each ear that was inoculated. The mean for each plant were calculated for each evaluated day. Photos were taken at 21 dpi for the overall measurment of type II resistance (Pathogen spread through the spike). The plants were then arranged based on susceptibility according to the disease severity scale (Engle *et al*., 2003).

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Molecular characterization

4.1.1. Screening the segregating F_1 base populations

The segregating F_1 base population of MS-MARS cycle 1 and 2 was screened (Addendum A and B) to determine the gene frequencies (Table 4.1) of*Sr2*, *Sr24*, *Sr26*, *Sr31*, *Lr19*, *Lr34* and *Lr37* respectively.

Table 4.1. Gene frequencies of MS-MARS cycle 1,2 and FHB donor material

Targeting genes	MS-MARS cycle 1	MS-MARS cycle 2
Sr2	40 %	42 %
Sr24	92 %	90 %
Sr26	0.8 %	0 %
Sr31	48 %	35 %
Lr19	1.68 %	0 %
Lr34	69 %	68 %
Lr37	9.45 %	23 %
Targeted QTL	**FHB donor material**	
Sr2	0 %	
Lr34	17 %	
Qfhs.ndsu-3BS	83.30 %	
7A QTL	17 %	
Qfhs.ifa-5A	66.67 %	

The low frequencies of *Sr26* in cycle 1 and the absence in cycle 2 can be explained, that *Sr26* is a recently introduced gene in the MS-MARS recurrent scheme and it will take a number of selection cycles for the gene frequencies to increase. According to Marais and Botes (2009) a minimum of four recurrent selection cycles are needed for the gene frequencies to increase significantly from 5 %. The absence in cycle 2 can be due to the random selection of only 60 screening plants. The*Lr19* gene frequencies are low, because the *Lr19*tranlocation in the population is linked to the unfavourable yellow pigment *Y* gene. The *Y* gene makes flour yellow which is unfavourable for baking properties. The *Lr19* translocation needs to be replaced with the *Lr19* translocation linked to *Sr25,* which does not cause a flour discolouration

4.1.2. Screening the male donor lines

Firstly, the FHB male donor lines were screened for *Sr2* and *Lr34*. Screening for slow rusting response genes is significant for the aim of the study. Secondly the FHB male donor lines were screened with the 9 SSR markers flanking the QTLs of interest (*Qfhs.ifa-5A*, *7A QTL* and *Qfhs.ndsu-3BS*). Gene frequencies were determined and listed in table 4.1. During each screening, 'SST027' was used as an unrelated negative control (not excluding the possibility of carrying FHB QTL) and 'Sumai 3' was used as a positive related control. 'SST027' showed polymorphic differences at the expected alleles and 'Sumai 3' amplified the expected allele sizes. 'SST027'did however amplify the allele for *Barc133*. One flanking marker for QTL validation can lead to false positives per Peng *et al*. (2000). For this reason, *BARC133* were scored as positive, but not linked to the related QTL. The amplification products for *Gwm133* were very light, which made scoring the amplification products extremely difficult. It was unreliable and therefore *Gwm133* together with *Gwm644* was not further used in this study. From the 9 SSR markers 7 were reliable and further applied in this study.

The male population in cycle 2 consisted of a selection of 8 lines from the 2015 SU-PBL nursery, 40 lines from the double haploid population and the 6 FHB donor lines used in cycle 1. The 2015 SU-PBL nursery was screened as part of an ongoing pre-breeding programme. The double haploid lines contained *Lr62/Yr42*, *Lr35/Yr35*, *Lr56/Yr38* and *Lr54/Yr37* rust species translocations, which was validated (Addendum C).

4.1.3. Multiplex attempt

Microsatellite markers or SSR's are one of the most abundant types of marker technology widely used by researchers for DNA analysis, construction of linkage maps, gene tagging etc (Butler *et al*., 2004). What make SSR's such a favourable choice is their multi-allelic, co-dominant and mendelian nature and reducibility through polymerase chain reaction (PCR). Unfortunately, the most abundant technique used for genotyping was the 6 % polyacrylamide gel electrophoresis (PAGE) stained with silver nitrate (Creste *et al*., 2001). This method was extremely time consuming and expensive especially when a large population needs to be screened individually.

Although PAGE gives better fragment separation compared to agarose gels it still take a lot of preparation time.

Genotyping technology has become more sophisticated with the introduction of capillary electrophoresis (CE). CE gives consistent and accurate allele sizes with minimal effort and reduces sizing error (Koumi *et al*., 2004). Successful studies have been reported in plants and one example was the one done in rice (*Oryzasativa*) (Nagaruju *et al*., 2002). It supported the cause for why it was significant to multiplex the seven markers flanking the FHB QTLs of interest in this study. It will reduce cost and labour intensity and will produce accurate and consistent allele sizes. The seven forward primers were labeled with fluorescent dyes at the 5' end (Chapter 3 table 3.5).

The samples were first evaluated on a 6 % PAGE to optimize the PCR conditions. After (PAGE) evaluation the seven flanking markers were genotyped individually at the central DNA sequencing Facility at Stellenbosch University (www.sun.ac.za/saf). The resistant check 'Sumai 3' and the susceptible check 'SST027' was used during the individual screening to score the expected allele sizes before performing the multiplex reaction. The following allele sizes for 'Sumai 3' and 'SST027' was observed in the electropherograms (Figure 4.1 and 4.2) respectively. 'Sumai 3' amplified the expected allele sizes. The only difference was *Gwm533* and *Gwm493*. These two markers amplified a 141 allele and a 194 allele respectively. The expected allele size for *Gwm533* and *Gwm493* was a 160 allele and a 211 allele per previous studies. Allele sizes of 141 and 195 had been observed in 'Sumai 3' according to Cuthbert *et al*. (2006). This lead to confirm the competency of the scoring results observed during agarose gel electrophoresis and CE. The comparison was significantly accurate and reassured the competency of the observed results in both agarose gel electrophoresis and CE. A few questions did however surface. The main question was; can the accuracy of one of these two techniques be responsible for a significant deviation from the actual allele size? This question was answered by a study done by Vemireddy *et al*. (2007) on the accuracy between PAGE, CE and agarose gels.

The study revealed significant differences in the observed allele sizes compared to the actual allele sizes. The averaged mean difference was calculated for each

electrophoresis type and the significant difference was observed in PAGE and agarose gels when compared to differences observed in CE. The averaged mean difference explained that CE only deviated (0.73 bp) from the actual allele size. For example if the actual allele size was 74 bp a slippage of only (73.27-74.73 bp) would be observed. The PAGE mean difference would create a deviation slippage of 5 bp (72-76 bp) and the cherry on the cake was the error observed in agarose gels with a 17 bp deviation from the actual allele size (66-82 bp) (Bligh, 2000, Jain *et al*., 2004, Saini *et al*., 2004 and Singh *et al*., 2004).

This study does not only explain the contradicting results in 'Basmati', but also explains why contradicting results in my study were observed for marker *Gwm533* and *Gwm493*. This defended the accuracy of the CE and why this multiplex approach carries significant value in producing accurate and reliable results that are not contradicting. Addendum E shows all the observed allele sizes according to 'Sumai 3' and 'SST027' for each of the lines tested.

4.2. The cross-pollination of the recurrent MS-MARS breeding scheme

4.2.1. MS-MARS cycle 1-2015

For the first cross-pollination (2014 SU-PBL nursery X F_1 base population) a total of 14 cutting sessions were performed for a period of 9 weeks. A total of 1437 male sterile and 1451 male fertile ears were sourced from the 1920 plants planted from both male sterile and male fertile populations. The male sterile plants were phenotypically selected based on the presence or absence of the dominant *Ms3*-gene located on chromosome 5 AS.

The presence was marked when the wheat anthers were unable to produce fertile pollen. The DNA responsible for producing fertile pollen was rearranged in the mitochondria. It's defined as a chimeric gene arrangement, this lead to the anthers inability of producing male fertile pollen (Hanson and Bentolila, 2004).

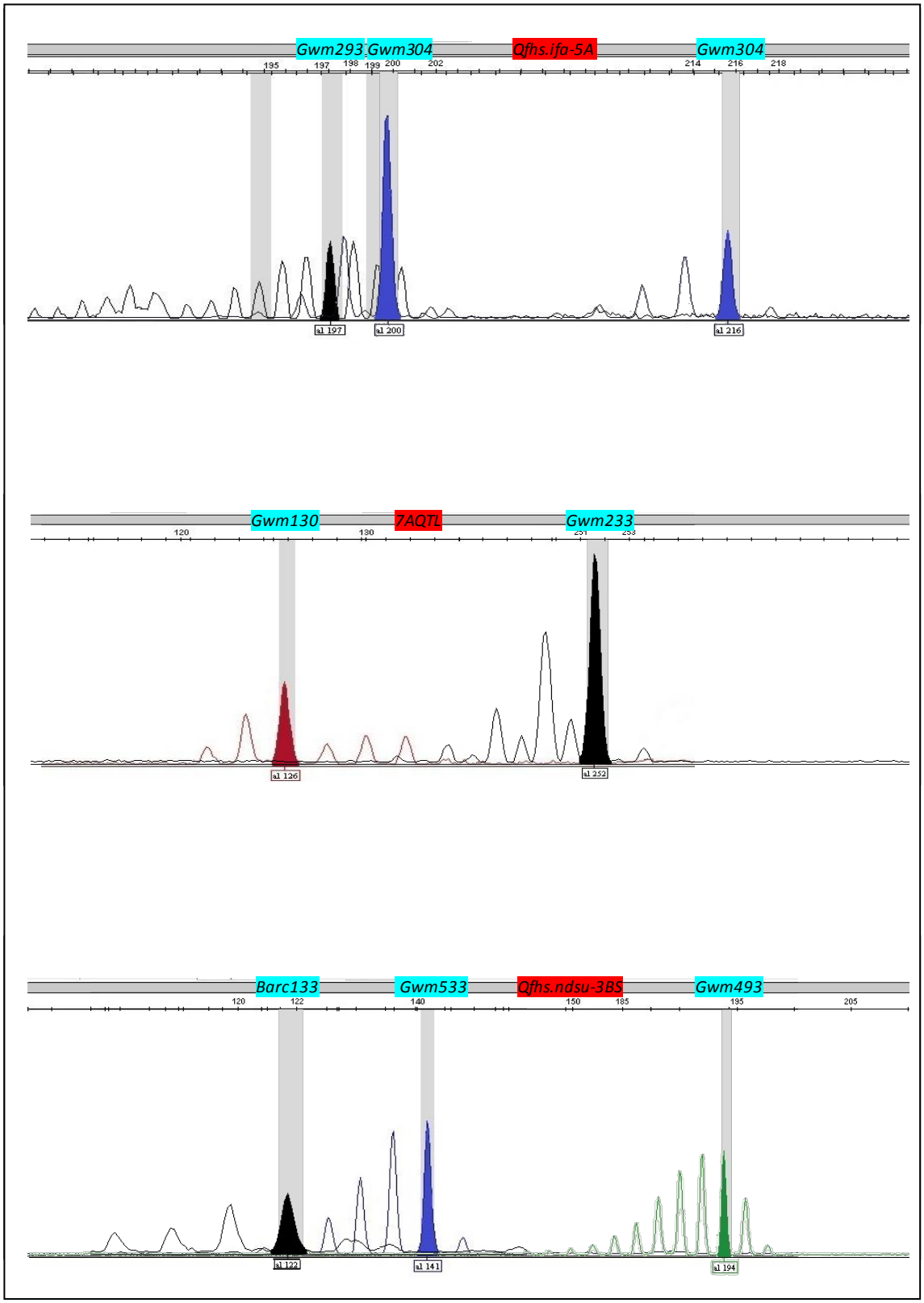


Figure 4.1. The electropherograms of the allele sizes observed in 'Sumai 3'

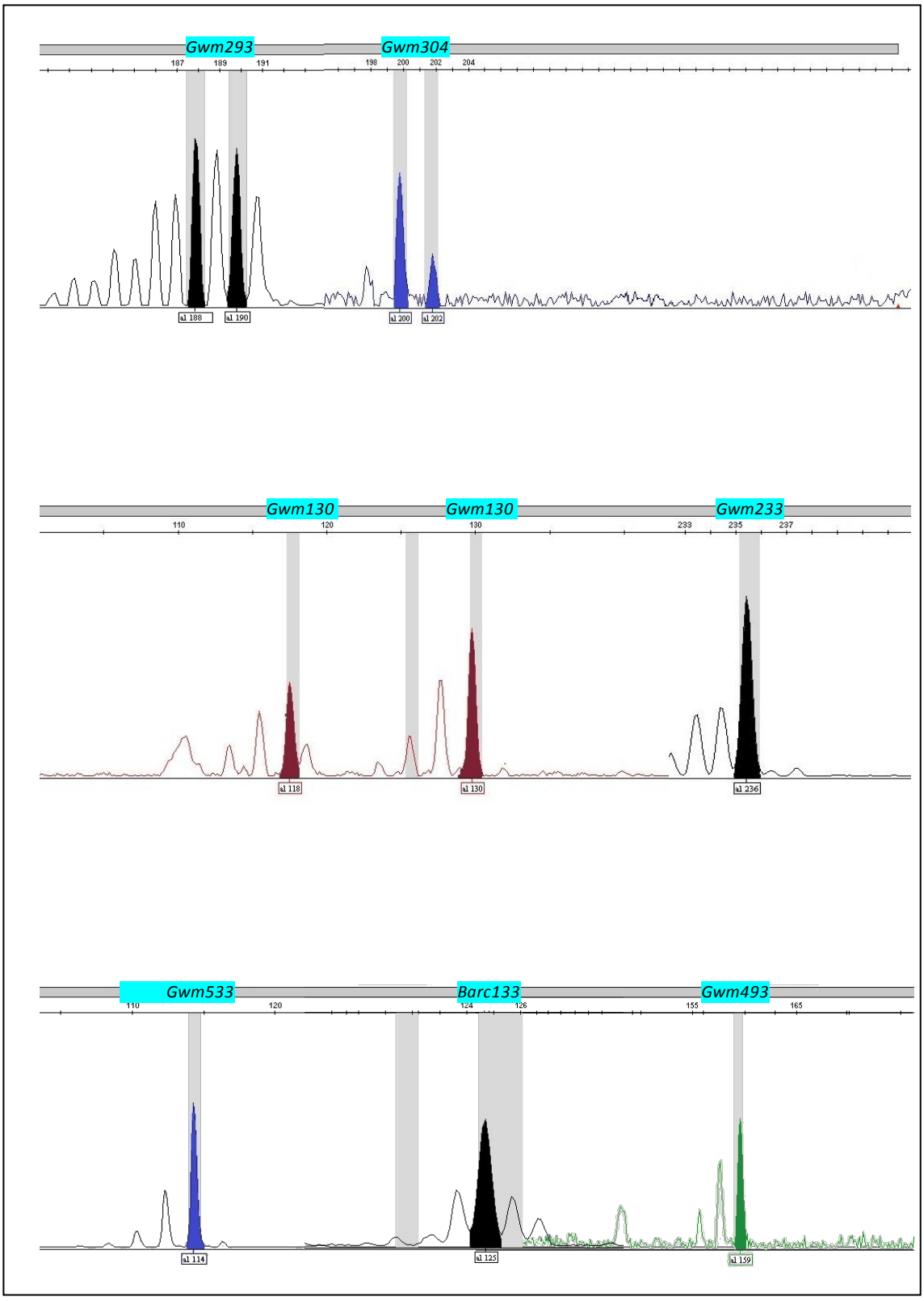


Figure 4.2. The electropherograms of the allele sizes observed in 'SST027'

A total of 1451 male sterile plants were successfully sourced and used as the male sterile population in recurrent cycle 1. From the male fertile population a total of 1437 plants were sourced and selected as pollen donors during recurrent cycle 1. In total 1415 male sterile plants from the 1451 successfully cross-pollinated producing 8616 shrivelled seed with an overall mass of 295 g. The shrivelling of the seed is the distinct characteristic distinguishing cross-pollinated plants from self-pollinated plants. This becomes an important selection parameter for obtaining pure cross-pollinated F_1 seed.

Table 4.2 list the cutting ratios of male fertile and male sterile plants for each executive week and the number of seed obtained. The chi-square for each of the tables was also calculated to determine the goodness of fit for a 1:1 male sterile, male fertile ratio (table 4.3). The second cross-pollination in cycle 1 was between the segregating F_1 base population and the FHB male donor lines. During every cutting session 10 male sterile and 20 male fertile ears were sourced from the segregating F_1 base population on bench 4 and from the FHB male donor lines. In total 627 F_1 seed were successfully cross-pollinated, harvested and used in the MS-MARS recurrent cycle 2.Figure 4.3 shows the arrangement of the cross-pollination cycles.

Figure 4.3. Shows the arrangement of the cross-pollination cycles

Table 4.2. MS-MARS cycle 1

week	male fertile	male sterile	possible combinations	harvest	Sterile plants sourced from	total seed/harvest	mass(g)
1	59	66	3894	1	229	1394	48
2	172	179	30788				
3	192	231	44352	2	444	2704	93
4	261	266	69426				
5	222	263	58386	3	452	2752	94
6	235	183	43005				
7	238	211	50218	4	290	1766	60
8	58	52	3016				
Total	**1437**	**1451**	**303085**		**1415**	**8616**	**295**

Table 4.3. Probability of fit

Table number	Sterile	Fertile	X^2	Probability of fit to a 1:1 ratio
1	213	207	0.085	0.77
2	219	213	0.082	0.77
3	228	209	0.83	0.36
4	242	215	1.59	0.2
Overall	**902**	**844**	**1.93**	**0.16**

Table 4.4. MS-MARS cycle 2

week	male fertile	male sterile	possible combinations	harvest	Sterile plants sourced from	total seed/harvest	mass(g)
1	55	97	5335	1	38	142	3.2
2	41	50	2050	2	13	64	1.6
3	42	48	2016	3	13	51	1.2
4	48	45	2160	4	10	14	0.5
5	44	41	1804	5	25	45	1
Total	**230**	**281**	**13365**		**99**	**316**	**7.5**

4.2.2. MS-MARS cycle 2-2015/2016

The second recurrent cycle was performed during the summer. Unfortunately extreme temperatures were experienced during the anthesis (reproductive and grain-filling) stage of the wheat plants. As a result the plants experienced heat stress. Heat stress occur when temperature exceed the optimum threshold for anthesis (15-22 °C), causing damage to the function and development of the plant (Hall, 2001).

The effect of heat stress can cause pollen sterility, tissue dehydration, low CO_2 assimilation and increased photorespiration (Fischer, 1980). Temperatures exceeding 30 °C during anthesis can cause complete sterility and reduce grain yield (Farooq *et al*., 2011). Based on the cited literature, the minimum and maximum temperatures were taken from accuweather. A graph was created from the raw weather data to specify whether the temperature threshold for the second recurrent cycle was exceeded (Figure 4.4).

It confirmed that the temperature threshold was indeed exceeded above optimum and that heat stress has led to the poor cross-pollination and low grain yield during cycle 2. Fischer (1985) reported a deduction of 4 % in grain number per spike for every 1 °C increase (from 15-22 °C). Saini and Aspinall, (1982) stated that wheat plants exposed to 30 °C for 3 days during anthesis, significantly reduces grain set. The hypothesis convincingly draws a parallel with the findings of Saini and Aspinall (1982) and Fischer (1985) for what could have causes the poor cross-pollination and low yield.

Despite the heat stress a cross-pollination cycle was achievable. Ten cutting sessions were performed over a period of five weeks. A total of 230 male sterile ears and 281 male fertile ears were sourced and selected from the two populations, producing 316 shrivelled F_1 seed with a mass of 7.5 g (Table 4.4). The seed was bulked with the F_1 2015 recurrent seed from cycle 1. The goodness of fit for the population was not calculated due to the heat stress situation. A few plants from pot 1 to 30 which were the plants from the F_1 FHB seed did self and produced F_2 seed.

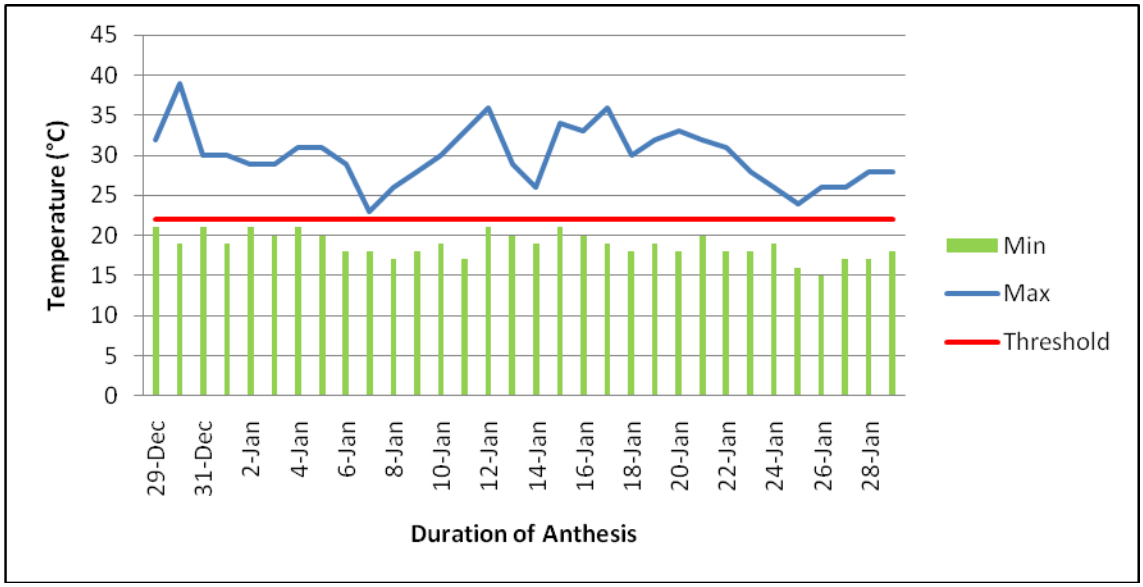


Figure 4.4. The minimum and maximum temperatures during the anthesis stage (AccuWeather, 2016)

4.2.3. Single seed decent (SSD)

The F_2 seed was harvested from the segregating F_1 base population of MS-MARS cycle 2. The seed was from the cross between the segregating F_1 base population and the FHB male donor lines. A total of 47 ears were sourced and harvested producing 853 F_2 seed with a mass of 18.9g. The seed was bulked and planted on the 30th of April 2016. The seed was planted in 3 L pots in numeric order from 1 to 96. Each pot contained 5 plants and was thinned out to 3 plants. Each plant was firstly individually screened for *Lr34* and *Sr2*. The gene frequencies of total number of plants containing *Lr34*, *Sr2* and a combination of both are shown in table 4.5. Secondly the plants with rust resistance was selected and screened for FHB resistant QTLs (*Qfhs.ifa-5A*, *7A QTL* and *Qfhs.ndsu-3BS*). A total of 170 plants with rust resistance were screened out of the 240 plants planted. A list of the selected plants was compiled containing rust genes and FHB resistance QTLs (Addendum D). The selected plants in addendum D were used for the phenotypic validation of type I and II resistance. The validation step was to confirm the presence of FHB resistance QTLs in the selected plants.

Table 4.5. Gene frequencies in SSD population

Targeted rust genes	Gene frequencies
Sr2	69 %
Lr34	21.13 %
Sr2/Lr34	9.86 %

4.2.4. Cloning the selected plants

The plants were first cut above the second node of the wheat stem and left for secondary growth. After two weeks 72 % secondary re-growth was achieved from the total plants cloned. Each plant was carefully removed from each pot and divided into separate cuttings. One were replanted in the original pot for SSD and the other cutting were planted in a new pot for type I and type II validation via a wool-point inoculation technique. This was done for all of the successfully cloned plants. The plants were left to reach maturity and at anthesis the wool-point inoculation technique was initiated. The number of inoculated ears depends on the number of regenerated ears per clone (three, two or one).

4.3. Phenotypic screening (QTL validation)

4.3.1. Wool-point inoculation (Type II resistance screening)

Two controls a resistant check 'Sumai 3' and a susceptible check 'SST027' was included in the screening trial. Disease severity was taken at 7, 10, 14 and 21 dpi (Figure 4.5-4.8). A digital photo at 21 dpi was taken to measure the overall disease level of each individual plant screened. To illustrate the disease severity, the control lines and selected plants are arranged in ascending severity (Figure 4.9). In addendum F all of the digital photos at 21 dpi are shown.

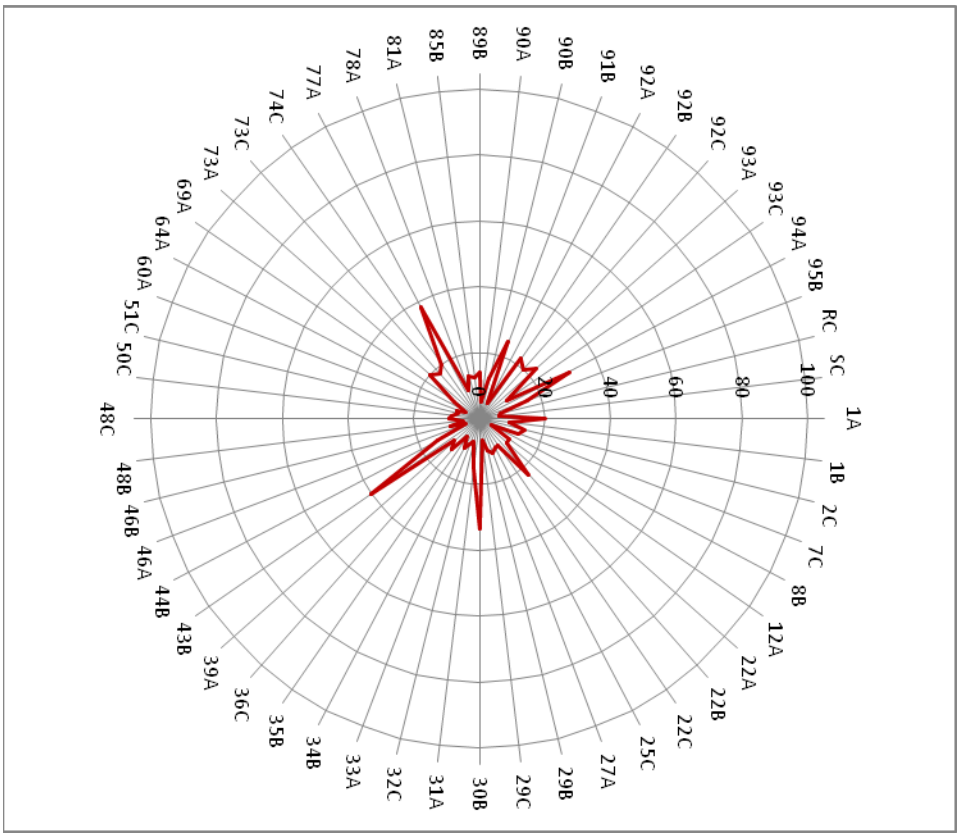


Figure 4.5. Disease severity at 7 dpi

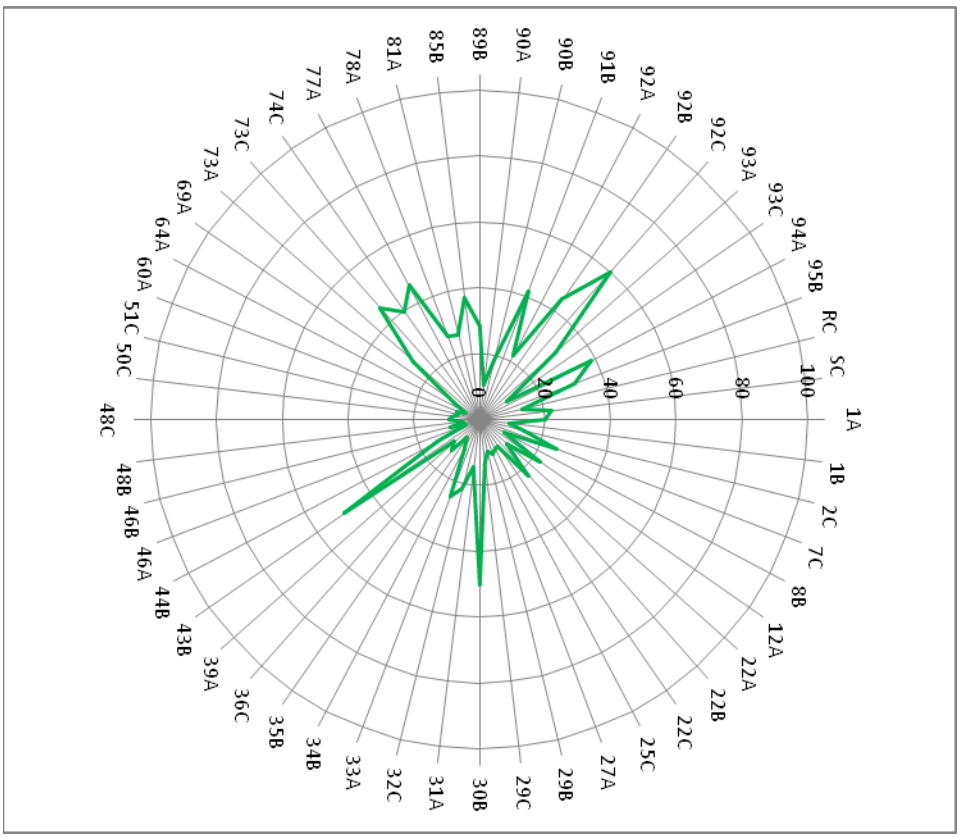


Figure 4.6. Disease severity at 10 dpi

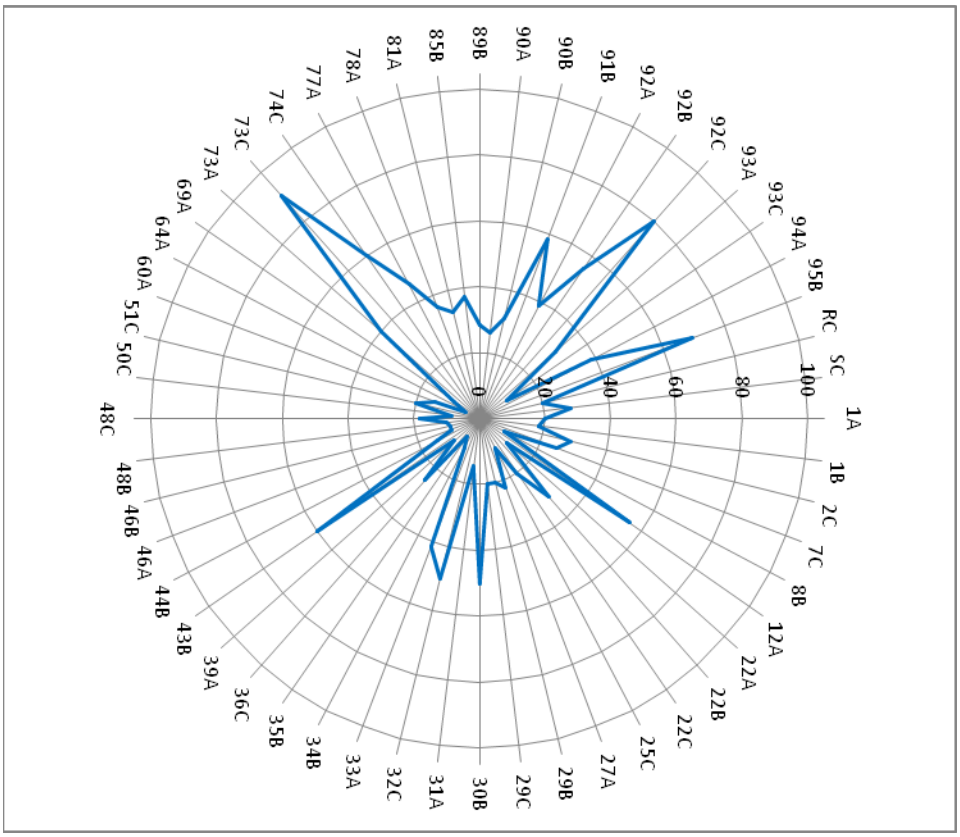


Figure 4.7. Disease severity at 14 dpi

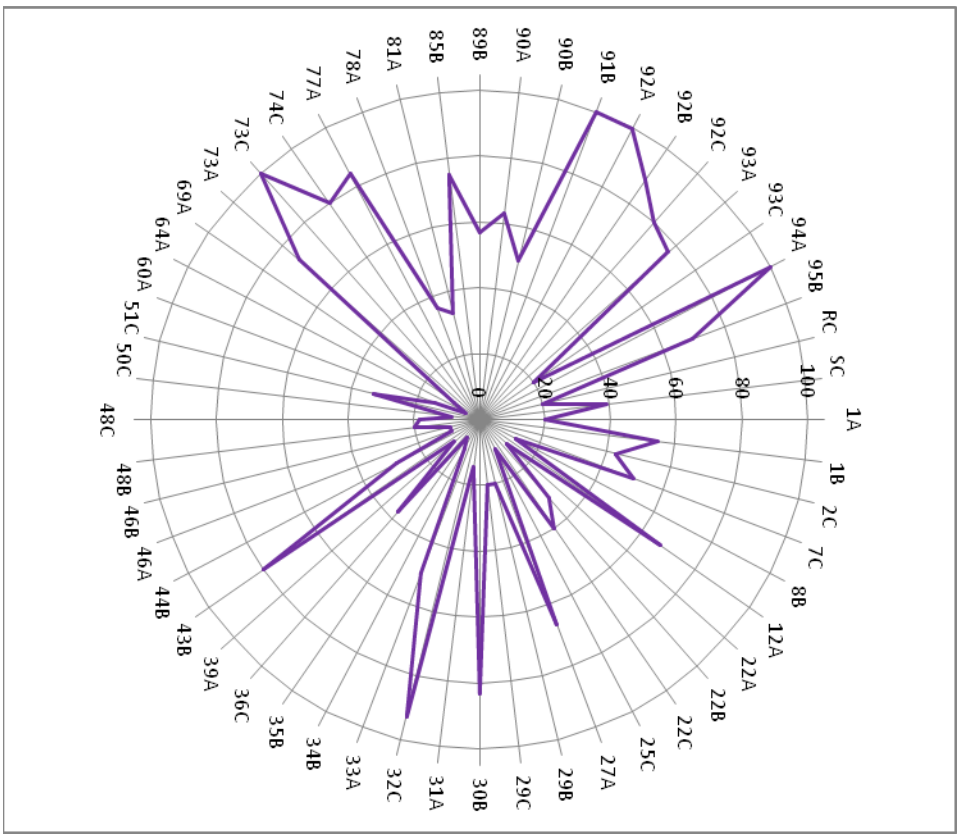
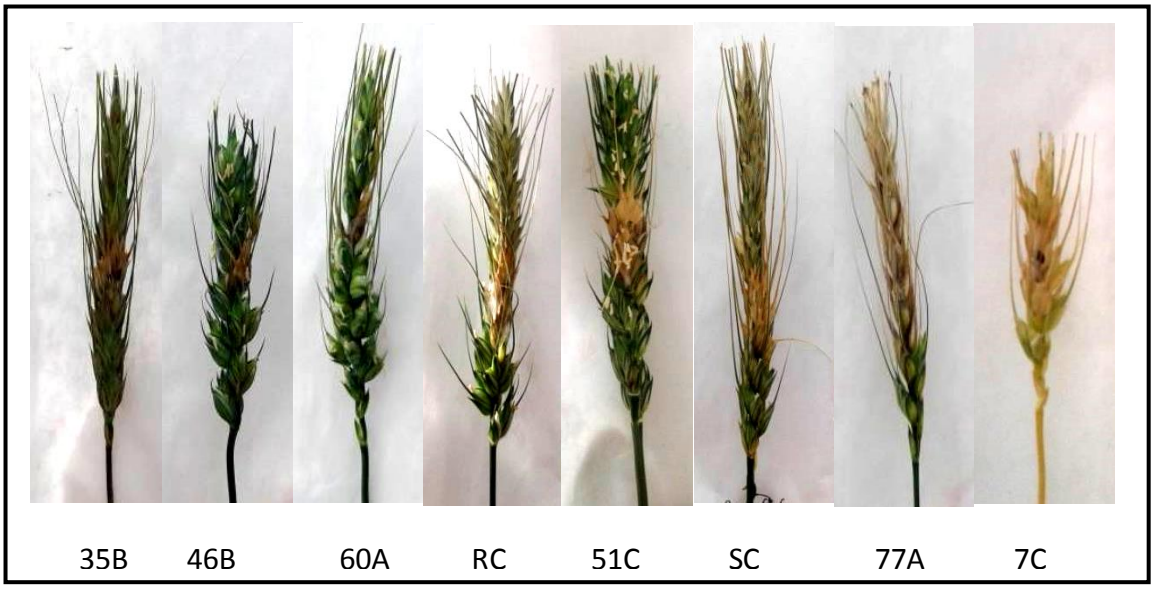


Figure 4.8. Disease severity at 21 dpi



The RC and SC represent the resistant check ('Sumai 3') and susceptible scheck ('SST027')

Figure 4.9. Illustrate the disease severity of controls and selected lines in ascending severity at 21 dpi

One occurrence that has been reported in many *F. graminearum* studies in wheat is the die back of the apical part of the wheat spike beyond the infection point. The *F. graminearum* develop papillae in the wheat rachis which cause a disruption in the phloem resulting in premature death beyond the infection point. This phenomenon has occurred in some of the inoculated plants in this study (46A, 93C, 'SST027' and 'Sumai 3'), which will eliminate some confusion when studying the photos in Addendum F. It did not limit the measurement of disease severity in these lines.

4.3.2. The genotypic/phenotypic interaction

The selected negative control lines were clear from any infection symptoms. The total number of florets for each line differed significantly ranging from 5 florets to 24 florets. The resistant check 'Sumai 3' performed significantly better when compared with the assigned susceptible check 'SST027'. 'Sumai 3' at 7 and 10 dpi spread to two florets and was stable onwards up to 21 dpi with only three florets in total being affected. Symptom spread was altered from 10 dpi. This clearly validated the presence of Type I and type II resistance (*Qfhs.ifa-5A, Fhb1*) in 'Sumai 3'. The following lines significantly outperformed 'Sumai 3' than what was expected.

Line 35B performed the best with only a 7 % disease severity at 21 dpi. Unfortunately, the only successful marker that amplified in 35B was *Gwm533*. All the other markers failed to amplify. *Gwm533* is a maker that flanks the (*Qfhs.ndsu-3BS*) QTL which is associated with symptom spread. This might explain the good suppression of symptom spread and confirms the presence of the (*Qfhs.ndsu-3BS*) QTL.

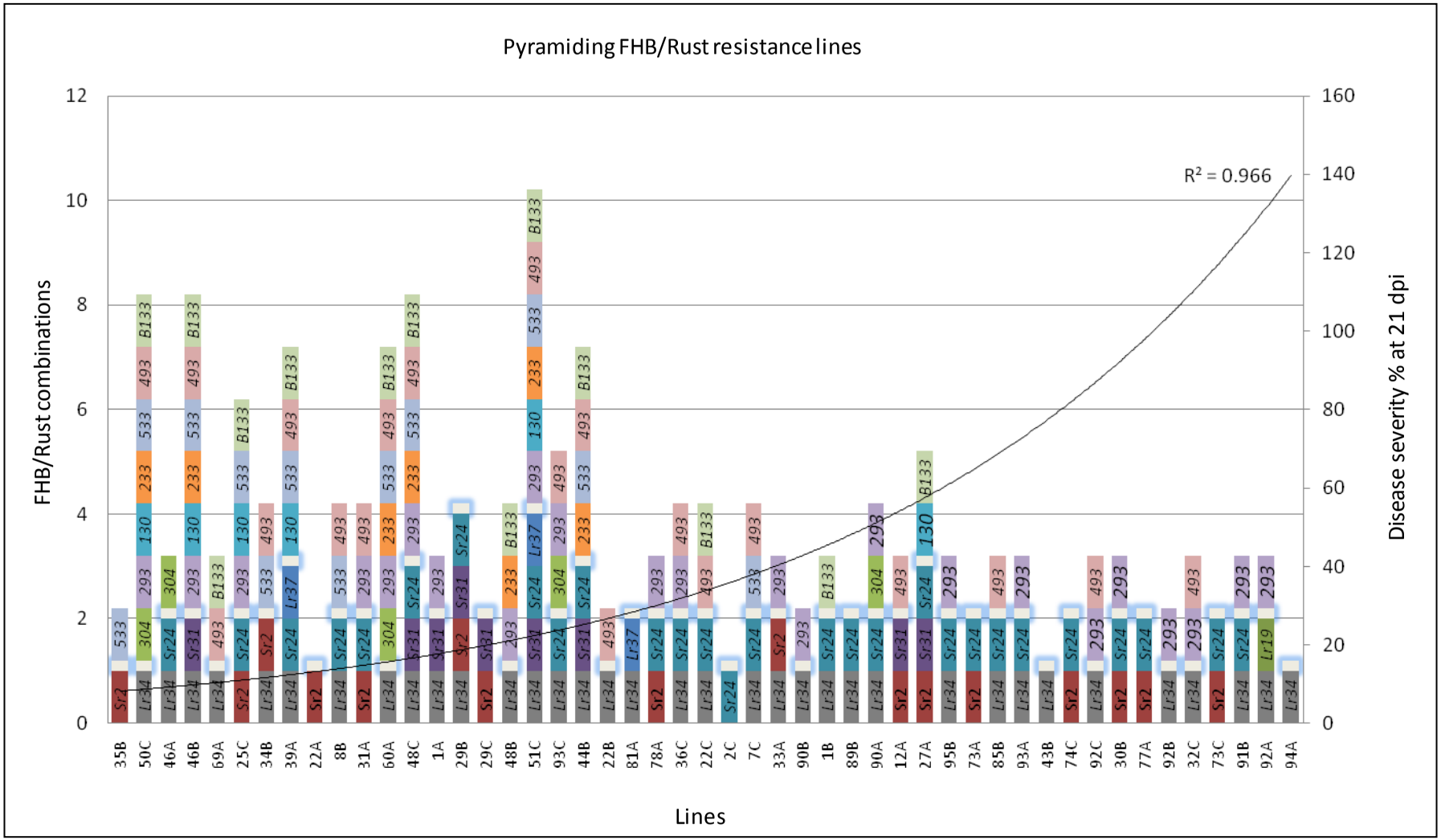
Line 50C amplified 6 out of the 7 markers with a disease severity of 8 % at 21 dpi. *Barc133* did not provide reliable results and was not included. Three lines 46B, 60A and 51C amplified 5 out of the 7 markers with disease severity 9 %, 14 % and 20 % respectively. Clearly the effect (*Qfhs.ndsu-3BS*) QTL has on symptom spread is evident in these significant lines.

The lines which performed the worst in comparison to 'Sumai 3' were 31 lines with disease severity ranging from 29 % to 100 %. A lack of type I and type II resistance was the cause for the poor performance of these lines. In addition, disease spread started before the 7 dpi measurement, in figure 4.5 line 43B had a disease severity of 40 % and lines 22B, 30B, 77A, 90B, 92B, 92C and 94C had ≥ 20 % disease severity at 7 dpi. The *Qfhs.ifa-5A*for type I resistance (pathogen penetration) was clearly absent in these lines. Only one marker (*Gwm293*) flanking the (*Qfhs.ifa-5A*) QTL amplified in 30B, 90B, 92B and 92C. What made this scenario different from line 35B was the spread of disease severity in these lines when compared to the disease severity in 35B. The severity was significant from the start of inoculation. This favoured the absence rather the presence of (*Qfhs.ifa-5A*) QTL.

Line 7C amplified both markers (*Gwm533* and *Gwm493*) flanking the (*Qfhs.ndsu-3BS) QTL*, but had a very high disease severity of 50 % at 21 dpi. One reason can be due to the total number of florets, in this case 7C had only 8 florets in total when compared to the other lines with up to 24 florets. The more florets there are the lower the disease severity will be. A hypothesis can be made, that if the (*Qfhs.ndsu-3BS) QTL* was absent all 8 florets would have been infected. In this case the observed marker data were significant for the presence of (*Qfhs.ndsu-3BS) QTL* and that symptom spread at 21 dpi only spread to 4 out of the 8 florets and would have been more if the (*Qfhs.ndsu-3BS) QTL* was absent. When the worst performing lines are compared with the best

performing lines it was clear that the best performing lines had either type I, type II or a combination of both type I and II. The indirect relationship between the disease severity observed and the total number of amplified FHB markers present in each line was significantly (Figure 4.10). The trend-line represents the disease severity progress curve at 21 dpi.

The selected SSD lines were additionally screened with the rust markers. The lines were screened for the presence of *Sr31*, *Sr24*, *Sr26*, *Lr37* and *Lr19*. The goal of this project was to pyramid a combination of rust genes and FHB QTLs in order to generate a pre-breeding line that can aid in resistance against these damaging fungal diseases in wheat. In figure 4.10 the selected lines are stacked according to each rust/FHB combinations. Most of the lines had *Lr34* which was a good starting point. Rust gene *Lr34* is an adult plant resistance gene and is associated with resistance to Powdery mildew (*Pm38*), leaf tip necrosis (*Ltn1*) and improved tolerance to barley yellow dwarf virus (*Bdv1*) (Keller *et al*., 2013). Lines 35B had a combination of *Lr34*, *Sr2* and *Fhb1*. This line alone can benefit a pre-breeding programme significantly. Line 51C was definitely a line to highlight as it contained the most rust/FHB combinations. Other lines of significance was 50C, 46B, 39A, 48C and 44B all these lines have*Lr34*. Line 50C amplified all of the FHB markers flanking the QTLs of interest.



( ) represent the link between rust and FHB resistance QTLs. The following FHB markers (GWM304, GWM293, GWM130, GWM233, GWM533, GWM493 and Barc133) are expressed as 304, 293, 130, 233, 533, 493 and B133 respectively. The rust markers are expressed as Lr34, Sr2, Sr24, Sr31, Lr19 and Lr37. The trend-line represents the disease progress curve.

Figure 4.10. Pyramiding lines containing FHB/rust combinations

Chapter 5 CONCLUSIONS

The segregating F_1 base population of MS-MARS cycle 1 and 2 was successfully molecular characterized for the presence of leaf, stripe and stem rust resistance genes (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34*, *Lr37* and *Lr19*). The male donor population used in the first sub-population in cycle 1 was molecularly characterised by a previous study for the presence of rust resistance genes (Springfield, 2014). Only the FHB male donor population used for the second sub-population in cycle 1 were successfully molecularly characterised for the presence of FHB resistance QTLs (*Qfhs.ifa-5A*, *7A QTL* and*Qfhs.ndsu-3BS*) and rust resistance genes (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34*, *Lr37* and *Lr19*). Sixty randomly selected lines in the segregating F_1 base population of MS-MARS cycle 2 was successfully molecularly characterised using molecular techniques for the presence of rust resistance genes (*Sr2*, *Sr31*, *Sr24*, *Sr26*, *Lr34*, *Lr37* and *Lr19*).

An attempt to multiplex the FHB markers flanking the resistance QTLs in one reaction was successfully executed. The benefits are definitely valuable; results are much more refined, precise, less labour intensive and cost efficient compared to agarose and PAGE electrophoresis methods. It will speed up the molecular validation of big research project were a large population needs to be characterized for FHB resistance QTLs. The observed allele sizes correlated with published allele sizes. Although a lot of controversy was picked up regarding published and observed allele size. The accuracy of allele size was clearly an issue based on which electrophoresis method was used. This highlights the importance of using CE in future studies to eliminate the allele size disagreement. Accuracy is key for any breeding programme to succeed.

Cross-pollination for the MS-MARS cycle 1 and 2 were successfully executed despite the heat stress during MS-MARS cycle 2. A total of 1415 male sterile plants were successfully cross-pollinated with 1437 pollen donors during MS-MARS cycle 1. A 1:1 Male sterile: Male fertile ration in MS-MARS cycle 1 were significant with an overall goodness of fit of 0.16. Confirming the success of the *Ms-3* gene controlling a good (1:1) inheritance ratio. In MS-MARS cycle 2 a total of 230 male sterile plants were successfully cross-pollinated with 281 pollen donors. The effect high temperatures

have on wheat clearly correspond and relate to reported articles and must be taken in consideration when conduction a cross-pollination attempt in the summer.

Selfed plants from the segregating F_1 population in MS-MARS cycle 2 was harvested prior cross-pollination for the development of a SSD population. The SSD population was successfully molecular characterised first for the presence of rust resistance *Sr2* and *Lr34*. Only the lines containing *Lr34*, *Sr2* or a combination of both were successfully characterized for the presence of FHB resistance QTLs and additionally screened with the rest of the rust resistance genes (*Sr31*, *Sr24*, *Sr26*, *Lr37* and *Lr19*)

The presence of the FHB resistance QTLs in the selected SSD population was successfully validated by a wool-point inoculation technique. The inoculated SSD lines were successfully measured for type I (penetration) and II (spread) resistance by rating the disease severity at 7, 10, 14 and 21 dpi. Seven significant lines (35B, 39A, 44B, 46B, 48C, 50C, 51C) with pre-breeding potential have been validated.

The aim and objectives were achieved for this study. Rust resistance genes and FHB resistance QTLs were successfully genotyped and phenotyped into 36 SSD lines. These lines will be used as male donor crossing-parents in the existing MS-MARS.

Future work for FHB QTL validation would be to perform a second inoculation trial with two techniques instead of only one. The spray inoculation technique for field evaluation in combination with point inoculation in the glasshouse will significantly increase the validation value. One advantage spray inoculation has over point inoculation, the infection occur more natural.

Future works for the multiplex approach would be to re- validate the presence of the flanking FHB markers. One attempt to consider: perform uniplex reactions for each pair of flanking makers and then pool them together for CE analysis. This attempt will reduce primer-primer interactions and unspecific amplification products. It can be beneficial when annealing temperature differ; it will reduce the optimisation time and the struggle to optimise all the FHB markers in one multiplex reaction.

Future studies can develop a double haploid population with pyramid rust resistance and FHB QTL. The double haploid population can be analysed to determine if the FHB QTL negatively influence the bread making properties.

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Addendum A: MS-MARS recurrent cycle 1

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
1A	Nursery 2014	April 2015	1	1	0	0	0	0
1B	Nursery 2014	April 2015	1	1	0	0	0	0
1C	Nursery 2014	April 2015	1	1	0	0	0	0
1D	Nursery 2014	April 2015	1	1	0	0	0	0
2A	Nursery 2014	April 2015	0	1	0	0	0	1
2B	Nursery 2014	April 2015	1	0	0	0	0	0
2C	Nursery 2014	April 2015	1	1	0	1	1	0
2D	Nursery 2014	April 2015	1	1	1	0	0	1
3A	Nursery 2014	April 2015	1	1	1	0	0	0
3B	Nursery 2014	April 2015	1	1	0	0	0	1
3C	Nursery 2014	April 2015	1	1	0	0	0	1
3D	Nursery 2014	April 2015	0	1	0	0	1	0
4A	Nursery 2014	April 2015	1	1	1	1	1	1
4B	Nursery 2014	April 2015	1	1	1	1	1	1
4C	Nursery 2014	April 2015	1	0	1	1	1	1
4D	Nursery 2014	April 2015	1	1	1	0	1	0
5A	Nursery 2014	April 2015	1	1	1	0	1	1
5B	Nursery 2014	April 2015	1	1	0	0	0	0
5C	Nursery 2014	April 2015	1	1	1	0	1	0
5D	Nursery 2014	April 2015	0	1	1	0	0	1
6A	Nursery 2014	April 2015	1	1	0	0	0	1
6B	Nursery 2014	April 2015	1	1	0	0	0	0
6C	Nursery 2014	April 2015	0	1	0	0	0	0
6D	Nursery 2014	April 2015	0	1	0	0	0	0
7A	Nursery 2014	April 2015	1	1	0	0	0	0
7B	Nursery 2014	April 2015	1	1	1	0	0	0
7C	Nursery 2014	April 2015	1	1	0	0	0	0
7D	Nursery 2014	April 2015	1	1	0	0	0	1
8A	Nursery 2014	April 2015	1	1	0	0	0	1
8B	Nursery 2014	April 2015	1	1	0	0	0	0
8C	Nursery 2014	April 2015	1	1	0	0	0	0
8D	Nursery 2014	April 2015	1	1	0	0	0	0
9A	Nursery 2014	April 2015	1	1	1	0	0	1
9B	Nursery 2014	April 2015	1	1	1	0	0	0
9C	Nursery 2014	April 2015	1	1	0	0	0	0
9D	Nursery 2014	April 2015	1	1	0	0	0	1
10A	Nursery 2014	April 2015	1	1	0	0	0	0
10B	Nursery 2014	April 2015	1	1	0	0	0	0
10C	Nursery 2014	April 2015	1	1	0	0	0	0
10D	Nursery 2014	April 2015	1	1	0	0	0	0
11A	Nursery 2014	April 2015	1	0	0	0	0	0
11B	Nursery 2014	April 2015	1	1	0	0	0	1
11C	Nursery 2014	April 2015	1	1	0	0	0	0
11D	Nursery 2014	April 2015	1	1	0	0	0	0
12A	Nursery 2014	April 2015	1	1	0	0	0	0
12B	Nursery 2014	April 2015	1	1	0	0	0	1
12C	Nursery 2014	April 2015	1	1	0	0	0	1
12D	Nursery 2014	April 2015	1	1	0	0	0	0
13A	Nursery 2014	April 2015	1	1	0	0	0	1
13B	Nursery 2014	April 2015	1	1	1	0	0	1
13C	Nursery 2014	April 2015	1	1	0	0	0	0
13D	Nursery 2014	April 2015	1	0	1	0	0	0
14A	Nursery 2014	April 2015	1	1	0	0	0	0
14B	Nursery 2014	April 2015	1	1	0	0	0	1
14C	Nursery 2014	April 2015	1	1	0	0	0	0
14D	Nursery 2014	April 2015	1	1	0	0	0	0
15A	Nursery 2014	April 2015	1	1	0	0	0	0
15B	Nursery 2014	April 2015	1	1	0	0	0	0

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
15C	Nursery 2014	April 2015	0	1	1	0	0	1
15D	Nursery 2014	April 2015	1	1	0	0	0	0
16A	Nursery 2014	April 2015	1	1	0	0	0	0
16B	Nursery 2014	April 2015	0	1	1	0	0	0
16C	Nursery 2014	April 2015	1	0	0	0	0	0
16D	Nursery 2014	April 2015	0	0	0	0	0	0
17A	Nursery 2014	April 2015	0	1	0	0	0	1
17B	Nursery 2014	April 2015	1	0	0	0	0	0
17C	Nursery 2014	April 2015	1	1	1	0	0	1
17D	Nursery 2014	April 2015	0	1	0	0	0	0
18A	Nursery 2014	April 2015	1	1	0	0	0	1
18B	Nursery 2014	April 2015	1	1	0	0	0	0
18C	Nursery 2014	April 2015	0	1	0	0	0	0
18D	Nursery 2014	April 2015	1	1	0	0	0	0
19A	Nursery 2014	April 2015	1	1	0	0	0	0
19B	Nursery 2014	April 2015	0	1	0	0	0	1
19C	Nursery 2014	April 2015	1	1	0	0	0	1
19D	Nursery 2014	April 2015	1	1	1	0	0	0
20A	Nursery 2014	April 2015	1	1	0	0	0	1
20B	Nursery 2014	April 2015	1	1	0	0	0	0
20C	Nursery 2014	April 2015	1	1	0	0	0	0
20D	Nursery 2014	April 2015	1	1	0	0	0	1
21A	Nursery 2014	April 2015	1	1	0	0	0	0
21B	Nursery 2014	April 2015	1	1	0	0	0	1
21C	Nursery 2014	April 2015	0	1	0	0	0	1
21D	Nursery 2014	April 2015	1	1	0	0	0	0
22A	Nursery 2014	April 2015	1	0	0	0	0	0
22B	Nursery 2014	April 2015	0	0	0	0	0	0
22C	Nursery 2014	April 2015	1	1	0	0	0	0
22D	Nursery 2014	April 2015	1	1	0	0	0	0
23A	Nursery 2014	April 2015	1	1	0	0	0	1
23B	Nursery 2014	April 2015	1	1	0	0	0	0
23C	Nursery 2014	April 2015	1	1	0	0	0	0
23D	Nursery 2014	April 2015	0	1	0	0	0	0
24A	Nursery 2014	April 2015	0	1	0	0	0	0
24B	Nursery 2014	April 2015	1	0	0	0	0	0
24C	Nursery 2014	April 2015	0	1	0	0	0	1
24D	Nursery 2014	April 2015	1	1	1	0	0	0
25A	Nursery 2014	April 2015	0	1	0	0	0	0
25B	Nursery 2014	April 2015	1	1	0	0	0	0
25C	Nursery 2014	April 2015	0	1	0	0	0	1
25D	Nursery 2014	April 2015	1	1	0	0	0	0
26A	Nursery 2014	April 2015	0	1	0	0	0	0
26B	Nursery 2014	April 2015	1	1	1	0	0	1
26C	Nursery 2014	April 2015	1	1	0	0	0	0
26D	Nursery 2014	April 2015	1	0	0	0	0	0
27A	Nursery 2014	April 2015	0	1	0	0	0	0
27B	Nursery 2014	April 2015	1	1	0	0	0	0
27C	Nursery 2014	April 2015	0	1	0	0	0	0
27D	Nursery 2014	April 2015	1	1	0	0	0	0
28A	Nursery 2014	April 2015	1	1	0	0	0	0
28B	Nursery 2014	April 2015	0	1	0	0	0	0
28C	Nursery 2014	April 2015	1	1	0	0	0	0
28D	Nursery 2014	April 2015	1	1	0	0	0	0
29A	Nursery 2014	April 2015	1	1	0	0	0	0
29B	Nursery 2014	April 2015	1	1	0	0	0	0
29C	Nursery 2014	April 2015	1	1	0	0	0	1
29D	Nursery 2014	April 2015	1	1	0	0	0	1
30A	Nursery 2014	April 2015	1	1	0	0	0	1
30B	Nursery 2014	April 2015	0	1	0	0	0	1

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
30C	Nursery 2014	April 2015	1	1	0	0	0	0
31A	Nursery 2014	April 2015	0	0	0	0	0	0
31B	Nursery 2014	April 2015	1	1	0	0	0	1
31C	Nursery 2014	April 2015	1	1	0	0	0	1
31D	Nursery 2014	April 2015	1	1	0	0	0	0
32A	Nursery 2014	April 2015	1	1	0	0	0	1
32B	Nursery 2014	April 2015	1	1	0	0	0	0
32C	Nursery 2014	April 2015	0	1	0	0	0	0
32D	Nursery 2014	April 2015	1	1	0	0	0	0
33A	Nursery 2014	April 2015	1	1	0	0	0	0
33B	Nursery 2014	April 2015	1	0	0	0	0	0
33C	Nursery 2014	April 2015	1	1	0	0	0	0
33D	Nursery 2014	April 2015	1	0	0	0	0	0
34A	Nursery 2014	April 2015	0	1	0	0	0	0
34B	Nursery 2014	April 2015	1	1	0	0	0	0
34C	Nursery 2014	April 2015	1	1	0	0	0	0
34D	Nursery 2014	April 2015	1	1	0	0	0	1
35A	Nursery 2014	April 2015	1	1	0	0	0	0
35B	Nursery 2014	April 2015	1	1	0	0	0	0
35C	Nursery 2014	April 2015	1	1	0	0	0	0
35D	Nursery 2014	April 2015	0	1	0	0	0	0
36A	Nursery 2014	April 2015	1	1	0	0	0	0
36B	Nursery 2014	April 2015	1	1	0	0	0	1
36C	Nursery 2014	April 2015	1	1	0	0	0	0
36D	Nursery 2014	April 2015	1	1	0	0	0	0
37A	Nursery 2014	April 2015	1	1	0	0	0	0
37B	Nursery 2014	April 2015	1	1	0	0	0	1
37C	Nursery 2014	April 2015	0	1	0	0	0	1
37D	Nursery 2014	April 2015	1	1	0	0	0	0
38A	Nursery 2014	April 2015	1	1	0	0	0	0
38B	Nursery 2014	April 2015	1	1	0	0	0	0
38C	Nursery 2014	April 2015	0	1	0	0	0	0
38D	Nursery 2014	April 2015	1	1	0	0	0	0
39A	Nursery 2014	April 2015	0	1	0	0	0	0
39B	Nursery 2014	April 2015	1	0	0	0	0	0
39C	Nursery 2014	April 2015	1	1	0	0	0	0
39D	Nursery 2014	April 2015	1	1	0	0	0	0
40A	Nursery 2014	April 2015	1	1	0	0	0	1
40B	Nursery 2014	April 2015	1	1	0	0	0	0
40C	Nursery 2014	April 2015	1	1	0	0	0	0
40D	Nursery 2014	April 2015	1	1	0	0	0	0
41A	Nursery 2014	April 2015	1	1	0	0	0	0
41B	Nursery 2014	April 2015	0	1	0	0	0	1
41C	Nursery 2014	April 2015	1	0	0	0	0	1
41D	Nursery 2014	April 2015	1	1	0	0	0	1
42A	Nursery 2014	April 2015	0	1	0	0	0	0
42B	Nursery 2014	April 2015	1	1	0	0	0	0
42C	Nursery 2014	April 2015	1	1	0	0	0	0
42D	Nursery 2014	April 2015	1	1	0	0	0	0
43A	Nursery 2014	April 2015	1	1	0	0	0	0
43B	Nursery 2014	April 2015	1	0	0	0	0	0
43C	Nursery 2014	April 2015	0	1	0	0	0	1
43D	Nursery 2014	April 2015	0	1	0	0	0	0
44A	Nursery 2014	April 2015	1	1	0	0	0	0
44B	Nursery 2014	April 2015	1	1	0	0	0	1
44C	Nursery 2014	April 2015	1	1	0	0	0	1
44D	Nursery 2014	April 2015	1	1	0	0	0	0
45A	Nursery 2014	April 2015	0	1	0	0	0	0
45B	Nursery 2014	April 2015	1	1	0	0	0	0
45C	Nursery 2014	April 2015	1	1	0	0	0	0

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
45D	Nursery 2014	April 2015	1	1	0	0	0	0
46A	Nursery 2014	April 2015	0	1	0	0	0	1
46B	Nursery 2014	April 2015	0	1	0	0	0	1
46C	Nursery 2014	April 2015	0	1	0	0	0	1
46D	Nursery 2014	April 2015	1	1	0	0	0	0
47A	Nursery 2014	April 2015	0	1	0	0	0	0
47B	Nursery 2014	April 2015	1	0	0	0	0	0
47C	Nursery 2014	April 2015	1	1	0	0	0	0
47D	Nursery 2014	April 2015	0	1	0	0	0	1
48A	Nursery 2014	April 2015	1	1	0	0	0	0
48B	Nursery 2014	April 2015	1	1	0	0	0	1
48C	Nursery 2014	April 2015	1	1	0	0	0	0
48D	Nursery 2014	April 2015	1	1	0	0	0	1
49A	Nursery 2014	April 2015	1	1	0	0	0	0
49B	Nursery 2014	April 2015	1	1	0	0	0	1
49C	Nursery 2014	April 2015	1	0	0	0	0	0
49D	Nursery 2014	April 2015	1	1	0	0	0	1
50A	Nursery 2014	April 2015	0	1	0	0	0	1
50B	Nursery 2014	April 2015	0	1	0	0	0	1
50C	Nursery 2014	April 2015	1	1	0	0	0	1
50D	Nursery 2014	April 2015	1	1	0	0	0	0
51A	Nursery 2014	April 2015	1	1	0	0	0	0
51B	Nursery 2014	April 2015	1	1	0	0	0	1
51C	Nursery 2014	April 2015	1	1	0	0	0	0
51D	Nursery 2014	April 2015	1	1	0	0	0	0
52A	Nursery 2014	April 2015	1	1	0	0	0	1
52B	Nursery 2014	April 2015	1	1	0	0	0	0
52C	Nursery 2014	April 2015	1	1	0	0	0	1
52D	Nursery 2014	April 2015	1	1	0	0	0	0
53A	Nursery 2014	April 2015	1	1	0	0	0	0
53B	Nursery 2014	April 2015	0	1	0	0	0	0
53C	Nursery 2014	April 2015	0	1	0	0	0	1
53D	Nursery 2014	April 2015	1	1	0	0	0	1
54A	Nursery 2014	April 2015	0	1	0	0	0	0
54B	Nursery 2014	April 2015	0	1	0	0	0	0
54C	Nursery 2014	April 2015	1	1	0	0	0	1
54D	Nursery 2014	April 2015	1	1	0	0	0	0
55A	Nursery 2014	April 2015	1	1	0	0	0	0
55B	Nursery 2014	April 2015	1	1	0	0	0	0
55C	Nursery 2014	April 2015	1	1	0	0	0	0
55D	Nursery 2014	April 2015	1	1	0	0	0	0
56A	Nursery 2014	April 2015	1	1	0	0	0	0
56B	Nursery 2014	April 2015	1	1	0	0	0	1
56C	Nursery 2014	April 2015	1	1	0	0	0	0
56D	Nursery 2014	April 2015	1	0	0	0	0	1
57A	Nursery 2014	April 2015	1	1	0	0	0	0
57B	Nursery 2014	April 2015	1	1	0	0	0	0
57C	Nursery 2014	April 2015	1	1	0	0	0	1
57D	Nursery 2014	April 2015	1	1	0	0	0	1
58A	Nursery 2014	April 2015	1	1	0	0	0	0
58B	Nursery 2014	April 2015	0	1	0	0	0	0
58C	Nursery 2014	April 2015	1	1	0	0	0	0
58D	Nursery 2014	April 2015	1	0	0	0	0	0
59A	Nursery 2014	April 2015	1	1	0	0	0	0
59B	Nursery 2014	April 2015	1	1	0	0	0	1
59C	Nursery 2014	April 2015	1	1	0	0	0	0
59D	Nursery 2014	April 2015	1	0	0	0	0	0
60A	Nursery 2014	April 2015	1	1	0	0	0	0
60B	Nursery 2014	April 2015	1	1	0	0	0	0
60C	Nursery 2014	April 2015	1	1	0	0	0	0

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
60D	Nursery 2014	April 2015	1	1	0	0	0	1
61A	Nursery 2014	April 2015	1	1	0	0	0	0
61B	Nursery 2014	April 2015	1	1	0	0	0	0
61C	Nursery 2014	April 2015	1	1	0	0	0	0
61D	Nursery 2014	April 2015	0	1	0	0	0	1
62A	Nursery 2014	April 2015	1	1	0	0	0	0
62B	Nursery 2014	April 2015	1	1	0	0	0	1
62C	Nursery 2014	April 2015	1	1	0	0	0	1
62D	Nursery 2014	April 2015	1	1	0	0	0	1
63A	Nursery 2014	April 2015	0	1	0	0	0	0
63B	Nursery 2014	April 2015	1	1	0	0	0	0
63C	Nursery 2014	April 2015	1	1	0	0	0	0
63D	Nursery 2014	April 2015	1	1	1	0	0	0
64A	Nursery 2014	April 2015	1	1	0	0	0	0
64B	Nursery 2014	April 2015	1	1	0	0	0	0
64C	Nursery 2014	April 2015	1	1	0	0	0	0
64D	Nursery 2014	April 2015	0	1	0	0	0	1
65A	Nursery 2014	April 2015	1	1	0	0	0	0
65B	Nursery 2014	April 2015	1	1	0	0	0	1
65C	Nursery 2014	April 2015	1	1	0	0	0	0
65D	Nursery 2014	April 2015	1	1	0	0	0	0
66A	Nursery 2014	April 2015	1	1	0	0	0	1
66B	Nursery 2014	April 2015	1	0	0	0	0	0
66C	Nursery 2014	April 2015	1	1	0	0	0	0
66D	Nursery 2014	April 2015	0	1	0	0	0	1
67A	Nursery 2014	April 2015	1	1	0	0	0	0
67B	Nursery 2014	April 2015	1	1	0	0	0	1
67C	Nursery 2014	April 2015	1	1	0	0	0	1
67D	Nursery 2014	April 2015	1	1	0	0	0	1
68A	Nursery 2014	April 2015	1	1	0	0	0	0
68B	Nursery 2014	April 2015	1	1	0	0	0	0
68C	Nursery 2014	April 2015	1	1	0	0	0	0
68D	Nursery 2014	April 2015	1	0	0	0	0	0
69A	Nursery 2014	April 2015	0	1	0	0	0	1
69B	Nursery 2014	April 2015	1	1	0	0	0	0
69C	Nursery 2014	April 2015	1	1	0	0	0	0
69D	Nursery 2014	April 2015	0	1	0	0	0	1
70A	Nursery 2014	April 2015	0	1	0	0	0	0
70B	Nursery 2014	April 2015	1	1	0	0	0	1
70C	Nursery 2014	April 2015	1	1	0	0	0	0
70D	Nursery 2014	April 2015	1	1	0	0	0	1
71A	Nursery 2014	April 2015	1	1	0	0	0	0
71B	Nursery 2014	April 2015	1	1	0	0	0	1
71C	Nursery 2014	April 2015	1	0	0	0	0	1
71D	Nursery 2014	April 2015	0	1	0	0	0	1
72A	Nursery 2014	April 2015	0	1	0	0	0	0
72B	Nursery 2014	April 2015	1	1	1	0	0	1
72C	Nursery 2014	April 2015	0	1	0	0	0	1
72D	Nursery 2014	April 2015	1	1	0	0	0	1
73A	Nursery 2014	April 2015	0	1	0	0	0	0
73B	Nursery 2014	April 2015	1	1	0	0	0	0
73C	Nursery 2014	April 2015	0	1	0	0	0	1
73D	Nursery 2014	April 2015	0	1	0	0	0	0
74A	Nursery 2014	April 2015	1	1	0	0	0	0
74B	Nursery 2014	April 2015	1	1	0	0	0	0
74C	Nursery 2014	April 2015	1	1	0	0	0	0
74D	Nursery 2014	April 2015	0	1	0	0	0	0
75A	Nursery 2014	April 2015	1	1	0	0	0	1
75B	Nursery 2014	April 2015	0	1	0	0	0	1
75C	Nursery 2014	April 2015	1	1	0	0	0	1

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
75D	Nursery 2014	April 2015	1	1	0	0	0	1
76A	Nursery 2014	April 2015	1	1	0	0	0	1
76B	Nursery 2014	April 2015	0	1	0	0	0	1
76C	Nursery 2014	April 2015	0	1	0	0	0	1
76D	Nursery 2014	April 2015	0	1	0	0	0	1
77A	Nursery 2014	April 2015	1	1	0	0	0	1
77B	Nursery 2014	April 2015	1	1	0	0	0	1
77C	Nursery 2014	April 2015	0	1	0	0	0	1
77D	Nursery 2014	April 2015	1	1	0	0	0	1
78A	Nursery 2014	April 2015	1	1	0	0	0	1
78B	Nursery 2014	April 2015	0	1	0	0	0	1
78C	Nursery 2014	April 2015	1	1	0	0	0	1
78D	Nursery 2014	April 2015	1	1	0	0	0	1
79A	Nursery 2014	April 2015	1	1	0	0	0	1
79B	Nursery 2014	April 2015	1	1	0	0	0	0
79C	Nursery 2014	April 2015	0	1	0	0	0	1
79D	Nursery 2014	April 2015	1	1	0	0	0	1
80A	Nursery 2014	April 2015	1	1	1	0	0	0
80B	Nursery 2014	April 2015	1	1	1	0	0	0
80C	Nursery 2014	April 2015	1	1	1	0	0	1
80D	Nursery 2014	April 2015	1	1	0	0	0	1
81A	Nursery 2014	April 2015	0	1	1	0	0	0
81B	Nursery 2014	April 2015	1	1	0	0	0	1
81C	Nursery 2014	April 2015	0	1	1	0	0	1
81D	Nursery 2014	April 2015	0	1	1	0	0	0
82A	Nursery 2014	April 2015	0	1	0	0	0	0
82B	Nursery 2014	April 2015	0	1	0	0	0	0
82C	Nursery 2014	April 2015	1	1	0	0	0	0
82D	Nursery 2014	April 2015	1	1	0	0	0	1
83A	Nursery 2014	April 2015	0	1	0	0	0	0
83B	Nursery 2014	April 2015	1	1	0	0	0	1
83C	Nursery 2014	April 2015	1	1	0	0	0	0
83D	Nursery 2014	April 2015	1	1	0	0	0	1
84A	Nursery 2014	April 2015	0	1	0	0	0	1
84B	Nursery 2014	April 2015	1	1	0	0	0	1
84C	Nursery 2014	April 2015	1	1	0	0	0	0
85A	Nursery 2014	April 2015	1	1	0	0	0	0
85B	Nursery 2014	April 2015	1	1	0	0	0	1
85C	Nursery 2014	April 2015	1	1	0	0	0	1
85D	Nursery 2014	April 2015	0	1	0	0	0	1
86A	Nursery 2014	April 2015	1	1	0	0	0	1
86B	Nursery 2014	April 2015	1	1	0	0	0	1
86C	Nursery 2014	April 2015	0	1	0	0	0	0
86D	Nursery 2014	April 2015	0	1	0	0	0	1
87A	Nursery 2014	April 2015	1	1	0	0	0	0
87B	Nursery 2014	April 2015	1	1	0	0	0	0
87C	Nursery 2014	April 2015	1	1	0	0	0	0
87D	Nursery 2014	April 2015	0	1	0	0	0	0
88A	Nursery 2014	April 2015	1	1	0	0	0	1
88B	Nursery 2014	April 2015	0	1	0	0	0	1
88C	Nursery 2014	April 2015	0	1	0	0	0	1
88D	Nursery 2014	April 2015	0	1	0	0	0	1
89A	Nursery 2014	April 2015	1	1	0	0	0	1
89B	Nursery 2014	April 2015	1	1	0	0	0	0
89C	Nursery 2014	April 2015	1	1	0	0	0	0
89D	Nursery 2014	April 2015	1	1	0	0	0	1
90A	Nursery 2014	April 2015	0	1	0	0	0	0
90B	Nursery 2014	April 2015	1	1	0	0	0	0
90C	Nursery 2014	April 2015	1	1	0	0	0	0
90D	Nursery 2014	April 2015	1	1	0	0	0	0

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
91A	Nursery 2014	April 2015	1	1	0	0	0	0
91B	Nursery 2014	April 2015	1	1	0	0	0	0
91C	Nursery 2014	April 2015	0	1	0	0	0	0
91D	Nursery 2014	April 2015	0	1	0	0	0	1
92A	Nursery 2014	April 2015	1	1	0	0	0	1
92B	Nursery 2014	April 2015	1	1	0	0	0	0
92C	Nursery 2014	April 2015	1	1	0	0	0	0
93A	Nursery 2014	April 2015	1	1	0	0	0	1
93B	Nursery 2014	April 2015	1	1	0	0	0	0
93C	Nursery 2014	April 2015	1	1	0	0	0	0
93D	Nursery 2014	April 2015	1	1	1	0	0	0
94A	Nursery 2014	April 2015	1	1	0	0	0	0
94B	Nursery 2014	April 2015	1	1	0	0	0	0
94C	Nursery 2014	April 2015	1	1	0	0	0	1
94D	Nursery 2014	April 2015	0	1	0	0	0	1
95A	Nursery 2014	April 2015	1	1	0	0	0	1
95B	Nursery 2014	April 2015	1	1	1	0	0	1
95C	Nursery 2014	April 2015	1	1	0	0	0	0
95D	Nursery 2014	April 2015	1	1	0	0	0	0
96A	Nursery 2014	April 2015	1	1	0	0	0	1
96B	Nursery 2014	April 2015	1	1	0	0	0	1
96C	Nursery 2014	April 2015	1	1	0	0	0	1
96D	Nursery 2014	April 2015	1	1	0	0	0	0
97A	Nursery 2014	April 2015	1	1	0	0	0	0
97B	Nursery 2014	April 2015	1	1	0	0	0	0
97C	Nursery 2014	April 2015	1	0	0	0	0	0
97D	Nursery 2014	April 2015	1	1	0	0	0	0
98A	Nursery 2014	April 2015	1	1	0	0	0	1
98B	Nursery 2014	April 2015	1	1	0	0	0	0
98C	Nursery 2014	April 2015	0	1	0	0	0	0
98D	Nursery 2014	April 2015	1	1	0	0	0	1
99A	Nursery 2014	April 2015	1	1	0	0	0	0
99B	Nursery 2014	April 2015	1	1	0	0	0	1
99C	Nursery 2014	April 2015	1	1	0	0	0	1
99D	Nursery 2014	April 2015	1	0	0	0	0	1
100A	Nursery 2014	April 2015	1	1	0	0	0	0
100B	Nursery 2014	April 2015	0	1	0	0	0	1
100C	Nursery 2014	April 2015	1	1	0	0	0	1
100D	Nursery 2014	April 2015	1	1	0	0	0	0
101A	Nursery 2014	April 2015	0	1	0	0	0	1
101B	Nursery 2014	April 2015	0	1	0	0	0	0
101C	Nursery 2014	April 2015	0	1	0	0	0	0
101D	Nursery 2014	April 2015	1	1	0	0	0	0
102A	Nursery 2014	April 2015	1	1	0	0	0	1
102B	Nursery 2014	April 2015	1	1	0	0	0	1
102C	Nursery 2014	April 2015	0	1	1	0	0	1
102D	Nursery 2014	April 2015	1	1	0	0	0	0
103A	Nursery 2014	April 2015	0	1	0	0	0	0
103B	Nursery 2014	April 2015	0	1	0	0	0	1
103C	Nursery 2014	April 2015	0	1	0	0	0	0
103D	Nursery 2014	April 2015	1	1	0	0	0	1
104A	Nursery 2014	April 2015	1	1	0	0	0	1
104B	Nursery 2014	April 2015	1	1	1	0	0	1
104C	Nursery 2014	April 2015	1	1	0	0	0	1
104D	Nursery 2014	April 2015	1	1	0	0	0	1
105A	Nursery 2014	April 2015	1	1	0	0	0	1
105B	Nursery 2014	April 2015	0	1	0	0	0	1
105C	Nursery 2014	April 2015	1	0	0	0	0	0
106A	Nursery 2014	April 2015	1	1	1	0	0	1
106B	Nursery 2014	April 2015	1	1	1	0	0	0

Male sterile lines cycle 1			Rust resistance					
Screened lines	Type	Planting date	***Lr34***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
106C	Nursery 2014	April 2015	1	1	0	0	0	1
106D	Nursery 2014	April 2015	1	1	0	0	0	1
107A	Nursery 2014	April 2015	1	1	0	0	0	0
107B	Nursery 2014	April 2015	1	1	0	0	0	0
107C	Nursery 2014	April 2015	1	1	0	0	0	1
107D	Nursery 2014	April 2015	1	1	0	0	0	0
108A	Nursery 2014	April 2015	0	1	0	0	0	1
108B	Nursery 2014	April 2015	1	1	0	0	0	1
108C	Nursery 2014	April 2015	1	1	0	0	0	1
108D	Nursery 2014	April 2015	0	0	0	0	0	0
109A	Nursery 2014	April 2015	1	1	0	0	0	0
109B	Nursery 2014	April 2015	1	1	0	0	0	1
109C	Nursery 2014	April 2015	1	1	0	0	0	1
109D	Nursery 2014	April 2015	1	1	0	0	0	0
110A	Nursery 2014	April 2015	1	0	0	0	0	0
110B	Nursery 2014	April 2015	1	1	1	0	0	0
110C	Nursery 2014	April 2015	0	1	0	0	0	1
110D	Nursery 2014	April 2015	1	1	0	0	0	1
111A	Nursery 2014	April 2015	0	1	0	0	0	1
111B	Nursery 2014	April 2015	1	1	0	0	0	1
111C	Nursery 2014	April 2015	1	1	0	0	0	1
111D	Nursery 2014	April 2015	1	1	0	0	0	0
112A	Nursery 2014	April 2015	1	1	1	0	0	0
112B	Nursery 2014	April 2015	0	1	0	0	0	1
112C	Nursery 2014	April 2015	1	1	0	0	0	0
112D	Nursery 2014	April 2015	1	1	1	0	0	1
113A	Nursery 2014	April 2015	1	1	1	0	0	1
113B	Nursery 2014	April 2015	0	1	0	0	0	0
113C	Nursery 2014	April 2015	1	1	0	0	0	0
113D	Nursery 2014	April 2015	1	0	1	0	0	0
114A	Nursery 2014	April 2015	1	1	1	0	0	0
114B	Nursery 2014	April 2015	0	1	0	0	0	0
114C	Nursery 2014	April 2015	1	1	0	0	0	1
114D	Nursery 2014	April 2015	1	1	0	0	0	0
115A	Nursery 2014	April 2015	1	1	1	0	0	1
115B	Nursery 2014	April 2015	1	1	0	0	0	1
115C	Nursery 2014	April 2015	1	1	0	0	0	1
115D	Nursery 2014	April 2015	0	1	0	0	0	0
116A	Nursery 2014	April 2015	0	1	1	0	0	1
116B	Nursery 2014	April 2015	1	1	1	0	0	1
116C	Nursery 2014	April 2015	1	1	1	0	0	0
116D	Nursery 2014	April 2015	1	1	0	0	0	1
117A	Nursery 2014	April 2015	1	1	1	0	0	0
117B	Nursery 2014	April 2015	0	1	0	0	0	0
117C	Nursery 2014	April 2015	0	0	0	0	0	0
117D	Nursery 2014	April 2015	0	1	0	0	0	0
118A	Nursery 2014	April 2015	1	1	0	0	0	0
118B	Nursery 2014	April 2015	1	1	0	0	0	1
118C	Nursery 2014	April 2015	1	0	0	0	0	0
118D	Nursery 2014	April 2015	1	1	0	0	0	0
119A	Nursery 2014	April 2015	0	0	0	0	0	0
119B	Nursery 2014	April 2015	1	1	0	0	0	0
119C	Nursery 2014	April 2015	0	1	0	0	0	1
119D	Nursery 2014	April 2015	1	1	0	0	0	0
120A	Nursery 2014	April 2015	1	1	0	0	0	0
120B	Nursery 2014	April 2015	1	0	0	0	0	1
120C	Nursery 2014	April 2015	1	1	0	0	0	0
120D	Nursery 2014	April 2015	1	1	0	0	0	1

Addendum B: MS-MARS recurrent cycle 2

MS-MARS cycle 2 (2015)			Rust resistance						
Screened lines	Type	Planting date	***Lr34***	***Sr31***	***Sr24***	***Lr37***	***Sr26***	***Lr19***	***Sr2***
4A	F_1 FHB seed	27-Oct	1	0	1	0	0	0	1
15C	F_1 FHB seed	27-Oct	1	0	0	0	0	0	1
19B	F_1 FHB seed	27-Oct	1	0	1	0	0	0	1
22D	F_1 FHB seed	27-Oct	1	0	1	0	0	0	1
27A	F_1 FHB seed	27-Oct	1	0	1	0	0	0	1
30D	F_1 FHB seed	27-Oct	0	0	0	0	0	0	1
35A	F_1 2015 seed	27-Oct	0	0	1	0	0	0	1
41C	F_1 2015 seed	27-Oct	1	1	1	0	0	1	0
49C	F_1 2015 seed	27-Oct	0	0	1	0	0	0	0
55B	F_1 2015 seed	27-Oct	0	0	1	1	0	0	0
63A	F_1 2015 seed	27-Oct	1	0	0	0	0	0	0
69D	F_1 2015 seed	27-Oct	0	1	1	1	0	0	0
2B	F_1 FHB seed	5-Nov	0	0	1	0	0	0	0
7C	F_1 FHB seed	5-Nov	0	0	1	0	0	0	0
13A	F_1 FHB seed	5-Nov	1	0	1	0	0	0	1
20D	F_1 FHB seed	5-Nov	1	0	1	0	0	0	0
25A	F_1 FHB seed	5-Nov	1	1	1	1	0	0	0
32C	F_1 FHB seed	5-Nov	1	1	1	0	0	1	1
43C	F_1 2015 seed	5-Nov	1	1	1	0	0	0	1
51B	F_1 2015 seed	5-Nov	1	0	1	0	0	1	1
54A	F_1 2015 seed	5-Nov	0	1	1	0	0	0	0
59B	F_1 2015 seed	5-Nov	1	1	1	0	0	0	1
64D	F_1 2015 seed	5-Nov	1	0	1	0	0	0	0
70B	F_1 2015 seed	5-Nov	1	0	0	1	0	0	0
3A	F_1 FHB seed	9-Nov	1	0	1	0	0	0	1
8C	F_1 FHB seed	9-Nov	1	0	1	0	0	0	1
14D	F_1 FHB seed	9-Nov	0	0	1	0	0	0	1
21D	F_1 FHB seed	9-Nov	0	0	1	0	0	0	0
26C	F_1 FHB seed	9-Nov	1	1	1	0	0	0	0
29A	F_1 FHB seed	9-Nov	1	0	1	0	0	0	1
36D	F_1 2015 seed	9-Nov	1	1	1	1	0	0	0
42C	F_1 2015 seed	9-Nov	0	0	1	0	0	0	1
48D	F_1 2015 seed	9-Nov	0	0	0	0	0	0	0
56B	F_1 2015 seed	9-Nov	1	0	1	1	0	0	1
60B	F_1 2015 seed	9-Nov	1	0	1	0	0	0	0
65A	F_1 2015 seed	9-Nov	0	0	1	0	0	0	0
1B	F_1 FHB seed	13-Nov	0	0	1	0	0	0	1
6B	F_1 FHB seed	13-Nov	1	0	1	0	0	1	0
10A	F_1 FHB seed	13-Nov	0	0	1	0	0	0	0
16D	F_1 FHB seed	13-Nov	1	0	0	0	0	0	1
24B	F_1 FHB seed	13-Nov	1	0	1	0	0	1	1
31A	F_1 FHB seed	13-Nov	1	0	1	1	0	0	0
37C	F_1 2015 seed	13-Nov	1	0	1	0	0	0	1
44D	F_1 2015 seed	13-Nov	1	1	1	0	0	1	0
52C	F_1 2015 seed	13-Nov	1	1	1	1	0	0	0
53D	F_1 2015 seed	13-Nov	1	1	1	0	0	0	0
57A	F_1 2015 seed	13-Nov	0	0	1	0	0	1	0
62C	F_1 2015 seed	13-Nov	1	1	1	1	0	0	0
5A	F_1 FHB seed	17-Nov	1	0	1	1	0	0	1
11B	F_1 FHB seed	17-Nov	0	1	1	0	0	0	0
18D	F_1 2015 seed	17-Nov	1	1	1	1	0	0	0
23C	F_1 2015 seed	17-Nov	1	1	1	0	0	0	0
28D	F_1 2015 seed	17-Nov	1	1	1	0	0	1	0
33A	F_1 2015 seed	17-Nov	0	1	1	0	0	1	1
39A	F_1 2015 seed	17-Nov	0	1	1	0	0	0	1
46A	F_1 2015 seed	17-Nov	1	0	1	0	0	0	0
50C	F_1 2015 seed	17-Nov	1	1	1	1	0	0	0
58D	F_1 2015 seed	17-Nov	1	1	1	1	0	0	0
61A	F_1 2015 seed	17-Nov	1	0	1	0	0	1	0
67B	F_1 2015 seed	17-Nov	1	0	1	1	0	0	0

Addendum C: Male population of recurrent cycle 2

	Species					Rust resistance															FHB			
Entry	***Lr62/Yr42***	***Lr59***	***Lr53/Yr35***	***Lr56/Yr38***	***Lr54/Yr37***	***Lr19***	***Lr24***	***Lr26***	***Lr34***	***Lr37***	***Sr2***	***Sr24***	***Sr26***	***Sr31***	***Sr38***	***Yr9***	***Yr18***	***Yr17***	***Pm8***	***Pm38***	***Qfhs.ndsu-3BS***	***Qfhs.ifa-5A***	***Fhb2***	***7AQTL***
13VH1-9-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-11	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-27	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-23	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-73	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-41-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-48	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-12-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-8-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-26	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-25	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-25-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-25-2	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-38-4	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-13-4	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-6-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-44-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-16	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-32	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-42-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-24	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-77	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-17	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-9	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-8	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-12	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-13	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-38-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-40-1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-56	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-72	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-53	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-50	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-46	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-69	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-63	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-39	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-74	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13VH1-68	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sumai cross 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Sumai cross 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Sumai cross 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sumai cross 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Sumai cross 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Sumai#3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
15US1M057	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
15US1M058	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0
15US1M124	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0
15US1M086	0	0	0	0	0	0	1	1	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0
15US1M046	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
15US1M116	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	1	0	1	1	0	0	0	0	0
15US1M122	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
15US1M063	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	1	1	0	1	1	0	0	0	0

Addendum D: Cloned SSD lines

Cloned SSD lines				Rust resistance							FHB resistance						
Selected lines	Disease severity %	Type	Planting date	*Lr34*	*Sr2*	*Lr19*	*Sr31*	*Sr24*	*Sr26*	*Lr37*	*GWM304*	*GWM293*	*GWM130*	*GWM233*	*GWM533*	*GWM493*	*BARC133*
35B	7	FHB/Rust	30-Mar	0	1	0	0	0	0	0	ND	ND	ND	ND	1	ND	0
50C	8	FHB/Rust	30-Mar	1	0	0	0	0	0	0	1	1	1	1	1	1	1
46A	9	FHB/Rust	30-Mar	1	0	0	0	1	0	0	1	0	0	0	0	ND	0
46B	9	FHB/Rust	30-Mar	1	0	0	1	0	0	0	ND	1	1	1	1	1	1
69A	9	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	ND	0	ND	0	1	1
25C	10	FHB/Rust	30-Mar	0	1	0	0	1	0	0	0	1	1	0	1	0	1
34B	10	FHB/Rust	30-Mar	1	1	0	0	0	0	0	ND	0	0	0	1	1	0
39A	10	FHB/Rust	30-Mar	1	0	0	0	1	0	1	ND	0	1	0	1	1	1
22A	11	FHB/Rust	30-Mar	0	1	0	0	0	0	0	ND	ND	0	0	0	0	0
8B	13	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	ND	0	0	1	1	0
31A	14	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	1	0	0	0	1	0
60A	14	FHB/Rust	30-Mar	1	0	0	0	0	0	0	1	1	0	1	1	1	1
48C	18	FHB/Rust	30-Mar	1	0	0	1	1	0	0	ND	1	0	1	1	1	1
1A	20	FHB/Rust	30-Mar	1	0	0	1	0	0	0	ND	1	0	0	0	0	0
29B	20	FHB/Rust	30-Mar	1	1	0	1	1	0	0	0	0	0	0	0	0	0
29C	20	FHB/Rust	30-Mar	0	1	0	1	0	0	0	ND	ND	0	ND	0	0	0
48B	20	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	1	0	1	0	0	1
51C	20	FHB/Rust	30-Mar	1	0	0	1	1	0	1	ND	1	1	1	1	1	1
93C	20	FHB/Rust	30-Mar	1	0	0	0	1	0	0	1	1	0	0	0	1	0
44B	29	FHB/Rust	30-Mar	1	0	0	1	1	0	0	0	0	0	1	1	1	1
22B	32	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	ND	0	ND	0	1	0
81A	33	FHB/Rust	30-Mar	1	0	0	0	0	0	1	ND	ND	0	ND	0	0	0
78A	36	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	1	0	0	0	0	0
36C	38	FHB/Rust	30-Mar	1	0	0	0	1	0	0	0	1	0	0	0	1	0
22C	40	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	0	0	0	0	1	1
2C	43	FHB/Rust	30-Mar	0	0	0	0	1	0	0	ND	ND	0	ND	0	0	0
7C	50	FHB/Rust	30-Mar	1	0	0	0	1	0	0	0	0	0	0	1	1	0
33A	50	FHB/Rust	30-Mar	1	1	0	0	0	0	0	ND	1	0	0	0	0	0
90B	50	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	1	0	0	0	0	0
1B	55	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	0	0	0	0	0	1
89B	57	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	0	0	0	0	0	0
90A	63	FHB/Rust	30-Mar	1	0	0	0	1	0	0	1	1	0	0	0	0	0
12A	67	FHB/Rust	30-Mar	0	1	0	1	0	0	0	0	ND	0	0	0	1	0
27A	67	FHB/Rust	30-Mar	0	1	0	1	1	0	0	0	0	1	0	0	0	1
95B	69	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	1	0	0	0	0	0
73A	73	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	0	0	0	0	0	0
85B	75	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	ND	0	ND	0	1	0
93A	77	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	1	0	0	0	0	0
43B	80	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	ND	0	ND	0	0	0
74C	80	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	ND	0	ND	0	0	0
92C	80	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	1	0	0	0	1	0
30B	83	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	1	0	0	0	0	0
77A	85	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	0	0	0	0	0	0
92B	89	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	1	0	0	0	0	0
32C	93	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	1	0	0	0	1	0
73C	100	FHB/Rust	30-Mar	0	1	0	0	1	0	0	ND	0	0	0	0	0	0
91B	100	FHB/Rust	30-Mar	1	0	0	0	1	0	0	ND	1	0	0	0	0	0
92A	100	FHB/Rust	30-Mar	1	0	1	0	0	0	0	ND	1	0	0	0	0	0
94A	100	FHB/Rust	30-Mar	1	0	0	0	0	0	0	ND	0	0	0	0	0	0

ND: No Data

Addendum E: Multiplex approach

Selected lines	*GWM493*	*GWM533*	*BARC133*	*GWM233*	*GWM130*	*GWM304*	*GWM293*
1B	155	114	126	236	118/131	ND	188/190
2C	ND	114	126	ND	119/130	ND	ND
7C	155	141	126	236	119/130	202	188
8B	155	141	126	236	119/130	ND	ND
12A	155/194	114	126	ND	118/131	200	ND
22A	ND	141	126	ND	ND	ND	ND
22B	ND	141	126	ND	ND	ND	ND
22C	155/194	114/141	125	ND	119/130	ND	ND
25C	155	114/141	125/126	ND	118/126/130	200	197
27A	155	114	124/126	ND	118/125/130	200	190
29B	155	114	125	236	119/130	200	ND
29C	ND	114	126	ND	119/130	ND	ND
30B	155	114	125	236	119/130	ND	197
31A	155/194	114	125/126	236	118/130	ND	197
32C	155	114	125	ND	119/130	ND	197
33A	155	114	125	ND	119/130	ND	197
34B	155	114/141	125	ND	119/130	ND	190
35B	ND	141	126	ND	ND	ND	ND
36C	155	114	125	ND	119/130	202	197
39A	155/194	114/141	125	236	119/126/130	ND	ND
43B	ND	141	126	ND	ND	ND	ND
44B	155	141	125	236/252	118/130	200	188/190
46A	155	ND	125	ND	118/131	200/216	190
46B	155/194	141	124	252	118/125	ND	190/197
48C	155	141	125	252	118/130	ND	197
50C	155	141	126	252	119/126	200/216	188/190/197
51C	155	141	126	252	119/126	ND	190/197
60A	155	141	125	252	118/130	202	188/197
69A	155	ND	125	ND	130	ND	ND
73A	ND	114	126	ND	118/130	ND	188/190
73C	ND	114	126	236	118/130	ND	188/190
74C	ND	114	126	ND	ND	ND	ND
77A	155	114	126	236	118/130	ND	188/190
78A	155	114	126	236	118/130	ND	188/190/197
81A	ND	141	126	ND	ND	ND	ND
85B	ND	ND	126	ND	ND	ND	ND
89B	155	114	125	236	118/130	ND	188/190
90A	155	114	126	236	118/130	202/216	197
90B	155	114	125	236	118/130	ND	197
91B	155	114	126	236	119/130	ND	188/190/197
92A	ND	114	126	236	118/130	ND	188/190/197
92B	155	114	126	236	118/130	ND	188/197
92C	155	114	126	ND	118/130	ND	190/197
93A	155	114	126	ND	118/130	ND	188/190/197
93C	155/194	114	126	236	118/130	216	190/197
94A	155	114	126	ND	118/130	ND	188/190
95B	155	114	126	236	118/130	ND	197
NEG	-	-	-	-	-	-	-
'Sumai 3'	194	141	126	252	126	200/216	197
'SST027'	155	114	125	236	118/130	200/202	188/190

ND: No Data

Addendum F: The inoculated lines at 21 dpi in ascending disease severity

35B 50C 46A 46B 69A 25C 34B 39A

22A 8B 31A 60A 48C 1A 29B 29C